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Toxicity of Leachates

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16. ABSTRACT

This report represents a multidisciplinary effort to establish a data base for evaluation of the toxicity of extracts from solid wastes representative of various industries. Seventeen solid wastes and an arsenic-contaminated groundwater were studied. The solid waste samples were subjected to the extraction procedure proposed in the Federal Register on December 18, 1978 (43 FR 58956), and the resulting extracts were characterized.

Analytical chemical methodologies were evaluated to determine their suitability for the use in analysis of the waste extracts, and procedures were established for preparing organic concentrates from the extracts.

Screening assays for toxicity to aquatic organisms and terrestrial plants were evaluated for use in characterization of waste extracts. Short term in vitro mutagenicity bioassays were evaluated and applied to testing the waste extracts.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay among its components require a concentrated and integrated attack on the problem.

Research and development, that necessary first step in problem solving, involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environment Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources; to preserve and treat public drinking water supplies; and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; it is a most vital communications link between the researcher and the community.

This report presents results from a laboratory investigation conducted to establish a data base for evaluating the toxicity of extracts from solid wastes representative of a variety of industries. Analytical chemical methods, screening assays for aquatic toxicity and phytotoxicity, and short-term in vitro mutagenicity bioassays were evaluated and applied to waste extracts.

Francis T. Mayo, Director
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ABSTRACT

This report represents a multidisciplinary effort to establish a data base for evaluation of the toxicity of extracts from solid wastes representative of various industries.

Seventeen solid wastes and an arsenic-contaminated groundwater were studied. The solid waste samples were subjected to the extraction procedure proposed in the Federal Register on December 18, 1978 (43 FR 58956), and the resulting extracts were characterized.

Analytical chemical methodologies were evaluated to determine their suitability for use in the analysis of the waste extracts, and procedures were established for preparing organic concentrates from the extracts. None of the extracts contained appreciable organic material. The groundwater sample was heavily contaminated with o-nitroaniline. Six of the wastes had metal concentrations in excess of the standard proposed in Section 3001, Subtitle C of the Resource Conservation and Recovery Act (PL 94-580).

Screening assays for toxicity to aquatic organisms and terrestrial plants were evaluated for use in chracterization of waste extracts. Four extracts exhibited toxicity to aquatic organisms at the proposed limit dilution. Fourteen wastes showed phytotoxic effects distinguishable from control values.

Short-term in vitro mutagenicity bioassays were evaluated and applied to testing the waste extracts. Only the arsenic-contaminated groundwater possessed mutagenic activity.

This report was submitted in fulfillment of DOE-IAG-40-646-77, EPA-IAG-78-DX-0372 by the Oak Ridge National Laboratory under the sponsorship of the U. S. Environmental Protection Agency. This report covers the period April 1, 1978, to May 18, 1979, when work was completed.

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LIST OF ABBREVIATIONS

AAS	atomic absorption spectrometry
Ar	Aroclor
BAP	benzo(a)pyrene
EP	Extraction Procedure
EPA	U. S. Environmental Protection Agency
GC	gas chromatography
NOEC	"no observed effects" concentration
ORNL	Oak Ridge National Laboratory
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyl
φB	phenobarbital
RCRA	Resource Conservation and Recovery Act

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SECTION 1

INTRODUCTION

Under Subtitle C of the Resource Conservation and Recovery Act (RCRA) of 1976 (PL 94-580), the Environmental Protection Agency (EPA) is required to promulgate regulations for the management of hazardous waste. To assist the EPA in developing characteristics for identifying wastes posing a potential hazard to human health and the environment, the Oak Ridge National Laboratory (ORNL) has conducted studies on leaching of toxicants from various solid waste materials, analytical procedures for characterizing extracts of wastes, and screening bioassays for evaluating the toxicity of extracts. This final report summarizes work during the period April 1, 1978, through May 18, 1979. Experimental work during this reporting period in support of the Office of Solid Waste's development of the proposed Extraction Procedure (EP) (43 FR 58956) has concentrated on:

(1) extracting 17 wastes of diverse origin by use of the EP to delineate potential problem areas;

(2) evaluating various analytical methodologies selected during ORNL's earlier work to determine their suitability for analyzing wastes and waste extracts;

(3) evaluating methodologies for preparing concentrates of the organic materials present in the extracts suitable for mutagenicity testing;

(4) evaluating short-term in vitro mutagenicity bioassays;

(5) evaluating screening assays for toxicity to the aquatic organism Daphnia magna and terrestrial plants;

(6) determining the utility of these procedures by use of the extracts prepared from the 17 wastes and from a sample of groundwater contaminated with arsenic.

Prior to the reporting period of this report, scoping studies were conducted using distilled water extracts (prepared at the University of Wisconsin) from 10 wastes. In addition, extracts were prepared at ORNL from municipal refuse, sewage sludge, and an arsenic-contaminated sludge. Four extractants were used: distilled water, acetate buffer, HCl acidified water, and Netherlands mix. The results of initial chemical characterization and environmental and biological toxicity testing of these materials were presented in a draft report to the Office of Solid Waste on May 2, 1978. It was in light of this early experience that the program described in this final report evolved.

SECTION 2

SUMMARY AND CONCLUSIONS

A number of experiments were conducted using a selection of industrial wastes in order to assist the EPA in developing and evaluating the EP proposed on December 18, 1978 (43 FR 58956). The studies consisted of four parts:

(1) Conducting the EP on 17 industrial wastes to uncover any problems inherent in the proposed EP and to gain experience in its application on a variety of waste types.

(2) Evaluating available analytical methods and analyzing the EP extracts for a variety of contaminants including heavy metals and organic compounds.

(3) Conducting range-finding studies under the direction of EPA's Office of Solid Waste to operationally define the EP. These involved experiments on agitation techniques, methods of adjusting pH, and preliminary studies on toxicity of extractants.

(4) Developing and evaluating screening tests for use in evaluating EP extracts for additional properties of mutagenicity, aquatic toxicity, and phytotoxicity.

The chemical analysis work was designed to evaluate protocols for identifying and quantifying selected heavy metals and organic compounds identified by the EPA as indicators of hazard. For heavy metals, the methods employed were those described in Methods for the Analysis of Water and Wastes, EPA-600/4-79-020, U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH 45268. For organic compounds, a variety of methods were employed including XAD resin concentration and the Bellar-Lichtenstein sparging technique. A preliminary validation of the methodology for the inorganic species was obtained through an interlaboratory reproducibility study.

No significant problems were encountered in extracting or analyzing wastes for inorganic species. Due to either the ionic character of the extraction fluid (water acidified with acetic acid), the low concentration of organic species in the wastes examined, the open extractor used, or possible adsorption on the filter membranes, no appreciable organic material was found in the waste extracts.

In order to develop a short-term screening test suitable for use in determining the potential toxicity of waste extracts to aquatic organisms, acute and chronic toxicities to the cladoceran D. magna were studied. Acute tests determined the concentration of the EP extract that was lethal to the organisms during a 48-h exposure. Of the wastes

tested, only the extract from the soybean process cake and gasification waste No. 1 exhibited no significant acute toxicity.

Chronic assays were conducted at extract dilutions of 1:100 and 1:1000, and effect on organism fecundity was used as the measure of toxicity. Five criteria were utilized to determine reproduction effects in the chronic tests. The mean number of young and the number of young per brood produced by each adult were the two criteria found most consistently sensitive for identification of toxicity. Toxicity assessment of the EP extracts used the number of young produced. Toxic effects were noted for four of the wastes tested at either one or both concentrations.

Short-term phytotoxicity tests were studied for use in determining if use of irrigation water contaminated by leachate from the waste posed a potential hazard to food crops. Two types of tests were studied, root elongation using radish and sorghum seeds, and seedling growth using wheat and soybean seeds. For the root elongation (radicle length) test, a chamber was developed in order to permit the precise measurement of radicle length in a timely manner. The longer-term (2-week) seedling growth studies used root and shoot dry weight and plant length as the measures of growth. Studies were performed to determine the optimal growth medium, methods of test solution application, and toxic effects of the waste extracts. Of the 18 samples tested, 14 proved to be significantly different from the controls in at least one of the tests. However, any conclusion relative to potential toxicity must be tempered by the observation that the acetate used in the EP exhibited significant phytotoxicity. Also, in a field situation soil attenuation could affect the apparent toxicity of leachates. These leachates were tested in the absence of soil.

The proposed Section 3001 regulations make use of a variety of short-term mutagenicity assays in the delisting procedures. In order to determine their utility, three assays diagnostic for genetic activity were evaluated by use of the available waste extracts and the arsenic-contaminated groundwater. The assays studied and the types of genetic activity they are known to detect are:

(1) Salmonella/microsome — point mutation in bacteria
(2) Saccharomyces $\underline{can}^r/\underline{his}^+$ — gene mutations in a lower eukaryote
(3) Salmonella $\underline{uvrB}$ — repair of DNA damage.

Of the 18 samples examined, only the arsenic-contaminated groundwater possessed detectable mutagenic activity. This material elicited a positive response in both the Salmonella/microsome and Saccharomyces assays. The bacterial DNA repair assay did not respond to the arsenic-contaminated groundwater sample. With regard to the negative results obtained with the remaining wastes, the extracts and even the XAD concentrates were extremely deficient in organic character. Furthermore, inorganic mutagens, which might have been present, are not detectable by the assays studied.

This study has identified a number of areas in which further work is required. These are:

(1) Reproducibility of the EP. Preliminary studies indicate it has a coefficient of variation in the range of 2 to 94% for elements such as Ca, Cr, and Ni.

(2) Effect of sample storage on EP extract. A potential problem was noted with loss of sample moisture affecting EP extract results.

(3) Extractor-induced contamination. Experiments conducted to evaluate this potential problem found that the level of contamination is related to the pH of the extraction and the abrasiveness of the waste. However, the maximum contamination noted was at least an order of magnitude below the level at which a waste would be determined to be hazardous under the proposed regulations (43 FR 58956).

(4) Possible retention of organic compounds on the filter media during filtering of the waste or the extracted liquid prior to evaluation of the extract.

(5) Effect of various types of agitation on integrity of solid wastes undergoing extraction.

(6) Methods of determining the phytotoxicity and aquatic toxicity of substances leachable from solid wastes in the presence of acetate or methods of measuring leachability which would not pose the problem encountered with the presence of acetate.

SECTION 3

RECOMMENDATIONS

Based on our observations, ORNL makes the following recommendations.

(1) Sample and extract storage specifications should be added to the regulations, such as those recommended in Methods for the Analysis of Water and Wastes, U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH 45268, and in the draft report Handbook for Sampling Hazardous Waste, California Department of Health, Berkeley, CA.

(2) A nonmetallic extractor, such as one fabricated from either polytetrafluoroethylene or glass could be used to lessen the possibility of contamination occurring during waste extraction.

(3) Alternative extraction procedures should be evaluated to increase the aggressiveness of the EP toward organic substances. One possible candidate may be the steam distillation procedure examined during the course of this work.

(4) Authentic landfill leachates should be compared to laboratory-generated extracts in order to assess the environmental relevance of the EP.

(5) Because of the confounding effects that acetate has on phytotoxicity studies, a procedure to determine phytotoxicity should be developed that does not employ acetic acid. Additional plant species should be considered for incorporation in the phytotoxicity testing battery.

(6) Because the presence of acetate can stimulate reproduction of the D. magna and acetate could have synergistic or antagonistic effects, additional studies should be performed to determine the effects acetate has on aquatic organisms; if necessary, a procedure should be developed to correct these problems.

SECTION 4

SAMPLES AND EXTRACTION

Seventeen solid wastes were extracted by use of the proposed EP published in 43 FR 58956 (see Appendix A). These samples represent various industrial wastes and coal combustion and gasification residues. Table 1 contains a listing of the waste samples and extraction dates. The arsenic-contaminated groundwater sample was distributed for testing as-received and was not extracted by the EP. Detailed information regarding the chemical and physical characteristics of the wastes was not provided with the samples.

TABLE 1. EXTRACTION DATE AND EXTRACTOR MATERIAL FOR WASTE SAMPLES

Waste	Extraction date	Extractor material*
Arsenic-contaminated groundwater	—	—
Soybean process cake	10/18/78	1
Metal processing waste	10/27/78	1
Plater's waste	11/02/78	1
Raw shale	11/28/78	2
Retorted shale	11/30/78	2
Dye waste	12/05/78	2
Textile waste	12/08/78	2
Municipal sewage sludge	03/06/79	3
Power plant No. 1 fly ash	09/22/78	1
Power plant No. 1 bottom ash	10/20/78	1
Power plant No. 1 scrubber sludge	10/03/78	1
Power plant No. 1 treated scrubber sludge	03/31/79	3
Power plant No. 2 fly ash	03/13/79	3
Fluidized bed residue	02/07/79	3
Gasification waste No. 1	10/06/78	1
Gasification waste No. 2	10/19/78	1
Gasification waste No. 3	11/02/78	1

*1, Plexiglas vessel and stirrer; 2, Type 316 stainless steel vessel and stirrer; 3, Type 316 stainless steel vessel with Teflon stirrer.

REPRESENTATIVE SAMPLING

All waste samples received for testing were stored outdoors in an open shed. This was a safety precaution deemed necessary due to lack of information concerning the nature of the samples.

We do not assume the waste samples to be necessarily representative of their respective solid wastes or industrial sources. The samples were supplied by EPA through other sources, and those sources refused all requests for additional information on the samples. Because of an extended storage time, and no information on collection and storage factors such as collection procedures for the samples, date of collection, temperature changes, and possible sample-container interactions, the samples must be evaluated as-received solely on the basis of their individual characteristics as determined during the course of analysis and evaluation at this laboratory.

It is unreasonable to assume or predict changes (biological, chemical, and physical) that may have occurred in a sample over time. These potential changes, though, should be a consideration in the final version of the hazardous waste characterization procedure.

No universal procedure was applicable in obtaining a representative subsample of the wastes for extraction. The types of wastes to be tested ranged from a dry powder to sludge and slurry. Therefore, upon examination of each waste, a judgment was made as to how an aliquot of the sample would be taken. In most cases thorough mixing and quartering was utilized.

PRELIMINARY SAMPLE DATA AND COMMENTS

Preliminary laboratory data collected on the waste samples (Table 2) included:

(1) Solids content, given as a fraction of the weight of the sample after drying per as-received sample weight.

(2) General physical description, including color, odor, and consistency.

As indicated by the data, solids content was highly variable among samples, ranging from 1.0000 to 0.2436.

A problem was identified in the extraction protocol with regard to the moisture content of individual wastes. After initial separation of a waste into liquid and solid phases by the methods of filtration or centrifugation, a considerable fraction of the solid weight was, in some cases, bound liquid. For example, the apparent weight of a 100-g solid sample containing 25% moisture by weight is not comparable to 100 g of dry sample. Leachate contaminant concentrations may be affected by differing moisture

TABLE 2. SAMPLE CHARACTERISTICS AND TREATMENT

Waste	Solids content*	Physical state	Sample treatment before extraction
Soybean process cake	0.4090	dark grey; moist, soil-like consistency and odor	none
Metal processing waste	0.4105	blue-green; moist, clay-like consistency and odor	none
Plater's waste	0.2436	orange sludge topped with large amount of orange liquid	supernatant removed by centrifugation prior to extraction, solid:liquid ratio = 0.606:1 (by wt.)
Raw shale	0.9991	grey; hard granular particles, heterogeneous mixture of large and small particles with the majority being <3/8-in., odorless	sized to pass 3/8-in. sieve
Retorted shale	0.9997	black; fine powder; odorless	none
Dye waste	0.4249	purple; moist, clay-like consistency	none
Textile waste	0.7053	light grey gelatinous solid with small amount of standing white liquid; fibers and small fabric pieces were mixed with waste; obnoxious odor	supernatant removed prior to extraction, solid:liquid ratio= 7:1 (by wt.)
Municipal sewage sludge	0.7359	dark brown; moist, soil-like consistency	none
Power plant No. 1 fly ash	0.9923	dark grey; powder-like; dead insects and dried plant materials mixed with waste; odorless	none
Power plant No. 1 bottom ash	0.7442	dark grey; heterogeneous mixture of large and small particles; odorless	sized to pass 3/8-in. sieve
Power plant No. 1 scrubber sludge	0.6844	dense, light grey sludge topped with small amount of standing liquid; cement-like odor	supernatant removed, solid:liquid ratio = 6.5:1 (by wt.)
Power plant No. 1 treated scrubber sludge	0.7803	dark grey sludge; clay-like consistency; odorless	none
Power plant No. 2 fly ash	0.9983	grey; powder-like; odorless	none
Fluidized bed residue	0.9999	light and dark grey; gravel-like particles; odorless	none
Gasification waste No. 1	0.9938	charcoal grey; fine with some larger agglomerates present	none
Gasification waste No. 2	1.0000	black; glassy, irregularly shaped particles with sharp edges	none
Gasification waste No. 3	0.9999	charcoal grey and brown; irregularly shaped particles; large agglomerates present	sample sized to pass 3/8-in. sieve

*Weight of sample after drying per original weight of sample.

contents and will not necessarily remain constant under varying conditions of collection time, transportation, and storage, due to evaporation.

EP AND COMMENTS

The extractions were executed by use of various extractor designs and materials. Designs and materials were progressively changed as problems arose.

Initially, the extractions were performed with four Plexiglas extractors borrowed from the Environmental Engineering Department of the University of Tennessee. Due to the possible problems of abrasion and solubility in organic media associated with the use of Plexiglas, EPA recommended the use of Type 316 stainless steel materials.

Extractors constructed of this material were then supplied to ORNL by EPA. Type 316 stainless steel, a high quality steel, was considered an improvement because of its inherent strength and resistance to abrasion, a factor which is especially important with hard, granular wastes such as bottom ash. The design was similar to the Plexiglas extractor and is given in 43 FR 58956. The suitability of the extractor was pretested with a hard, granular solid. This type of waste had presented stirring problems in the Plexiglas extractor. Similar problems occurred with this prototype. The major problems included: binding of the stirring blade and solid particles, movement of the vessel, stalling of the stirring motor, uneven blade alignment, and sample grinding. This unit was not used for any of the reported sample extractions.

As a result of these problems, a modified version of the extraction equipment was designed and fabricated at ORNL, incorporating EPA's recommendations. As requested by EPA, all parts exposed to waste or extract were fabricated of Type 316 stainless steel. Details of materials and design are given in Appendix B, Figures B-1 to B-11. As shown in the figures, the most important improvement in the modified design is the unitizing of vessel, supporting frame, and stirring motor. The integration of the vessel, support stand, stirring rod, and stirring motor is viewed as the best way of assuring positive alignment of the agitator blade and vessel bottom. This feature minimizes the probability of waste particles binding and assures a smooth mixing action. A high-torque, low-rpm stirring motor and its accompanying solid-state controller have proven more than adequate for the job and provide positive, variable-speed propulsion of the stirring rod. The conical bearing surface for the stirring rod on the bottom of the vessel is an important improvement because it assures positive centering of the stirring rod in the vessel and minimizes grinding action and clogging between the stirring rod end and the vessel bottom. This unitized system allows close tolerances to be maintained between the edges of the stirring blade and the bottom and sides of the extraction vessel, thereby assuring thorough mixing of the solid and liquid phases during the EP.

The new extractor design eliminated many of the previous problems. However, the main problem remaining was that of sample grinding (particle size reduction) when the particle size was approximately 1/4 in. Extractor designs having a fixed blade near

the vessel bottom would be expected to permit sample grinding. The spacing would determine which particle size is susceptible to this action. To eliminate sample grinding, the secured stainless steel blade was replaced with a Teflon stirrer shaft with two moveable blades that swing out when rotated (Cole-Palmer cat. no. 6367-42). The Teflon stirrer blade permitted similar stirring action, eliminated sample grinding, and avoided binding. Table 1 contains a listing of the extractor design used for each waste sample.

An inherent problem associated with the use of a Type 316 stainless steel extractor is the dissolution of metals from the vessel wall and stirring blade. Due to the low concentrations of contaminant being analyzed, a noncontaminating extractor material should be utilized. An extractor material such as Type 316 stainless steel has the potential of introducing metals such as Cr, Ni, and Zn into the extract. The extent of this contamination is discussed below under Extraction Procedure Blanks and in Section 5. To avoid the problem of extract contamination by extractor material, it is recommended that Teflon or glass materials be used.

Due to the lack of specification in the EPA proposed EP, the laboratory practices and utilization of reagents cited in Appendix B were deemed adequate. We recommend the specification of water and chemical grades as an addition to the EP.

EXTRACTION DATA AND COMMENTS

Data recorded during the extraction of the solid wastes are represented in Table 3. The following parameters were recorded for each individual extraction apparatus:

(1) initial pH,
(2) final pH,
(3) amount of 0.5 N acetic acid initially added to adjust the solution pH to 5,
(4) total 0.5 N acetic acid added during the 24-h extraction period,
(5) electrical conductivity of the final extract.

The solution pH was automatically adjusted during all extractions.

EXTRACT DATA AND COMMENTS

Following extraction, the solid and liquid phases were separated by use of a Millipore filtration system. A Millipore (Type HA) 0.45-μm pore size filter was utilized for final filtration. In all cases, vacuum filtration proved to be satisfactory for all wastes extracted to date. Research activities concerning the filtration of EP extracts can be found at the end of this section.

Data recorded upon completion of the extractions are given in Table 4. The following parameters were recorded for the extract in its final form:

TABLE 3. EXTRACTION DATA

Waste	Extraction (or mean for extractions)	Initial pH (1:16 solid: solution ratio)	Final pH (1:16 solid: solution ratio)	Acetic acid initially added (meq/g sample extracted)	Total acetic acid added (meq/g sample extracted)	Electrical conductivity (μmhos/cm)
Soybean process cake	1	8.2	5.0	0.063	0.077	284
	2	8.6	5.1	0.071	0.082	265
	3	8.6	5.1	0.063	0.077	235
	4	8.6	5.2	0.062	0.073	255
	mean	8.5	5.1	0.065	0.077	260
Metal processing waste	1	9.4	5.2	1.37	1.47	2,780
	2	9.4	5.2	1.29	1.46	3,290
	3	9.3	5.2	0.94	1.38	2,950
	4	9.6	5.2	1.17	1.34	2,950
	mean	9.4	5.2	1.19	1.41	2,993
Plater's waste	1	11.4	5.6	2.0*	2.0*	4,620†
	2	11.4	5.5	2.0*	2.0*	4,900†
	3	11.4	5.4	2.0*	2.0*	5,000†
	4	11.4	5.6	2.0*	2.0*	5,100†
	mean	11.4	5.5	2.0	2.0	4,815
Raw shale	1	10.0	5.1	0.50	1.45	3,650
	2	10.0	5.1	0.37	1.36	3,450
	3	9.85	5.1	0.64	1.50	3,800
	4	9.9	5.1	0.43	1.18	3,350
	mean	9.9	5.1	0.49	1.37	3,562.5
Retorted shale	1	11.45	8.3	2.0*	2.0*	5,700
	2	11.45	8.4	2.0*	2.0*	5,750
	3	11.3	8.3	2.0*	2.0*	5,720
	4	11.4	8.6	2.0*	2.0*	5,750
	mean	11.4	8.4	2.0	2.0	5,730
Dye waste	1	9.9	5.1	0.30	0.32	1,900
	2	9.9	4.9	0.72	0.72	2,200
	3	9.9	5.1	0.68	0.68	2,000
	4	9.9	4.9	0.26	0.41	1,600
	mean	9.9	5.0	0.49	0.53	1,925

(Continued)

(Table 3 continued)

Waste	Extraction (or mean for extractions)	Initial pH (1:16 solid: solution ratio)	Final pH (1:16 solid: solution ratio)	Acetic acid initially added (meq/g sample extracted)	Total acetic acid added (meq/g sample extracted)	Electrical conductivity (μmhos/cm)
Textile waste	1	8.8	6.7	2.0*	2.0*	5,200†
	2	8.7	7.5	2.0*	2.0*	5,200†
	3	8.7	7.5	2.0*	2.0*	5,200†
	4	8.75	7.6	2.0*	2.0*	5,200†
	mean	8.7	7.3	2.0	2.0	5,200
Municipal sewage sludge	1	6.6	5.1	0.03	0.25	1,570
	2	6.5	5.1	0.03	0.23	1,660
	3	6.5	5.2	0.03	0.25	1,610
	4	6.5	5.1	0.03	0.24	1,613
	mean	6.5	5.1	0.03	0.24	1,613
Power plant No. 1 fly ash	1	4.5	5.0	0.00	0.156	2,000
	2	5.5	4.9	0.05	0.207	1,980
	3	5.0	4.9	0.00	0.254	1,960
	4	5.0	5.0	0.00	0.146	2,000
	mean	4.9	4.9	0.01	0.191	1,985
Power plant No. 1 bottom ash	1	9.6	5.1	0.011	0.026	141
	2	9.4	5.2	0.010	0.027	151
	3	9.2	5.2	0.010	0.023	150
	4	9.2	5.1	0.011	0.026	162
	mean	9.3	5.1	0.011	0.026	151
Power plant No. 1 scrubber sludge	1	9.8	5.1	0.415	0.490	3,380†
	2	9.8	5.1	0.413	0.478	3,070†
	3	10.1	5.1	0.420	0.516	3,270†
	mean	9.9	5.1	0.416	0.495	3,240
Power plant No. 1 treated scrubber sludge	1	7.2	5.0	—	0.759	—
	2	7.1	5.1	—	0.736	—
	3	7.1	5.05	—	0.673	—
	4	7.1	5.05	—	0.691	—
	mean	7.1	5.05	—	0.715	—

(Continued)

(Table 3 continued)

Waste	Extraction (or mean for extractions)	Initial pH (1:16 solid: solution ratio)	Final pH (1:16 solid: solution ratio)	Acetic acid initially added (meq/g sample extracted)	Total acetic acid added (meq/g sample extracted)	Electrical conductivity (μmhos/cm)
Power plant No. 2 fly ash	1	4.5	5.1	0.00	0.03	480
	2	4.7	5.1	0.00	0.04	469
	3	4.85	4.9	0.00	0.06	476
	mean	4.7	5.0	0.00	0.04	475
Fluidized bed residue	1	12.5	12.35	2.0*	2.0*	11,000
	2	12.5	12.55	2.0*	2.0*	11,000
	3	12.5	12.6	2.0*	2.0*	12,000
	mean	12.5	12.5	2.0	2.0	11,333
Gasification waste No. 1	1	4.9	4.9	0.00	0.00	240
	2	5.1	5.1	0.00	0.00	254
	3	5.2	5.2	0.00	0.00	261
	4	5.0	5.0	0.00	0.00	246
	mean	5.0	5.0	0.00	0.00	250
Gasification waste No. 2	1	4.7	4.8	0.00	0.00	26
	2	4.5	4.8	0.00	0.00	41
	3	4.5	4.7	0.00	0.00	44
	mean	4.6	4.8	0.00	0.00	37
Gasification waste No. 3	1	9.6	5.2	0.023	0.063	305
	2	9.4	5.2	0.022	0.052	270
	3	9.5	5.1	0.022	0.044	220
	4	9.6	5.1	0.023	0.057	288
	mean	9.5	5.1	0.0225	0.054	271

*Maximum amount allowed (4 ml acid/g solid).

†Supernatant not added.

TABLE 4. EP EXTRACT DATA

Waste	pH	Electrical conductivity (μmhos/cm)	Total acetic acid added (meq/g sample)	Color	Remarks
Soybean process cake	5.20	240	0.077	transparent, yellow	
Metal processing waste	5.11	3,100	1.41	transparent, colorless	
Plater's waste	6.95	7,000	1.22*	translucent, orange	precipitate immediately formed after addition of the supernatant; the precipitate was filtered out, and the eluate, precipitate, and supernatant were all sent to Analytical Chemistry Division
Raw shale	5.15	3,300	1.37	transparent, colorless	
Retorted shale	7.53	5,750	2.0*	transparent, colorless	
Dye waste	5.03	1,600	0.53	opaque, dark blue	
Textile waste	7.02	5,200	1.75*	translucent, white	
Municipal sewage sludge	5.07	1,620	0.24	transparent, yellow	
Power plant No. 1 fly ash	4.80	1,910	0.191	transparent, colorless	
Power plant No. 1 bottom ash	5.00	154	0.030	transparent, colorless	
Power plant No. 1 scrubber sludge	4.87	2,340	0.429	transparent, colorless	white precipitate formed after addition of supernatant to leachate; the precipitate was filtered out, and the precipitate and the filtrate were sent separately to Analytical Chemistry Division
Power plant No. 1 treated scrubber sludge	5.0	2,600	0.715	transparent, colorless	
Power plant No. 2 fly ash	4.9	480	0.04	transparent, colorless	
Fluidized bed residue	12.11	10,000	2.0*	transparent, colorless	
Gasification waste No. 1	5.05	190	0.00	transparent, colorless	
Gasification waste No. 2	4.88	33	0.00	transparent, colorless	
Gasification waste No. 3	5.19	193	0.054	transparent, colorless	

*Maximum amount allowed (4 ml acid/g solid).

(1) pH,
(2) electrical conductivity,
(3) total 0.5 N acetic acid added,
(4) color,
(5) remarks on unusual appearance or occurrence.

After the EP was completed, aliquots of the extract were delivered to appropriate groups at ORNL for analysis. When delivery could not be accomplished immediately after completion of the procedure, all extract samples were refrigerated at 4°C.

EXTRACTION REPRODUCIBILITY

Sample extract variability was investigated between the extracting vessels utilized. Four 2.5-l vessels were utilized for each sample extraction to produce at least 8 l of combined sample EP extract. An aliquot of EP extract was reserved from each vessel before the extracts were combined and distributed for testing. These aliquots were analyzed separately for Cr, Ni, and Ca concentrations, to provide information concerning the variability associated with each extraction. Concentrations of Cr and Ni were investigated because of the possible contamination with these metals from the extracting vessel. Concentrations of Ca were determined because of the excellent sensitivity and reproducibility associated with analytical Ca concentration determinations.

The coefficient of variation was calculated for each sample investigated as a function of the Cr, Ni, and Ca concentrations analyzed (Table 5). (Coefficient of variation values normalize sample variation by expressing the standard deviation as a percentage of the mean, for comparison purposes.) As these values indicate, the variability ranged from 2 to 94%; however, no overall trends were evident.

Figures 1 to 3 graphically describe the high and low concentrations, mean, and reported values for the EP concentrations of Cr, Ni, and Ca. These figures generally indicate that the EP variability trend exceeds that expected for the analytical determinations. An investigation of the percent variabilities associated with analytical analysis is recommended.

The high degree of variability found for each sample extraction indicates the need for further research in the area of EP reproducibility. Possible explanations for sample-to-sample variation have been discussed in previous sections, such as the lack of information concerning sample collection and storage, and the solids content of the waste samples. The temperature, length of storage, type of container, and pH all have the potential of affecting the variability of extract contaminant concentrations. Further research is needed to investigate the sources and degree of variability associated with EP variables and extract analysis.

TABLE 5. SAMPLE EP EXTRACT CONCENTRATIONS OF CHROMIUM, NICKEL, AND CALCIUM BETWEEN VESSELS UTILIZED, EXPRESSED IN TERMS OF THE COEFFCIENT OF VARIATION*

Extract	Coefficient of variation (%) for		
	Cr	Ni	Ca
Soybean process cake	43.61	87.01	11.11
Metal processing waste	16.61	13.90	7.84
Plater's waste	53.06	28.03	13.95
Raw shale	63.70	38.27	9.43
Retorted shale	6.75	19.84	6.51
Dye waste	72.20	53.66	28.92
Municipal sewage sludge	2.36	9.57	2.56
Power plant No. 1 bottom ash	61.39	94.35	5.33
Power plant No. 1 scrubber sludge	12.80	19.10	4.43
Power plant No. 2 fly ash	82.59	11.18	4.23
Gasification waste No. 1	14.30	9.31	4.80
Gasification waste No. 2	60.70	47.30	27.86
Gasification waste No. 3	61.65	41.51	14.97

*Coefficient of variation = (S.D./mean) X 100.

EP BLANKS

As previously discussed Type 316 stainless steel for the extractor materials has the potential of contaminating EP extracts. Water blanks alone only provide indications of possible background contamination. Solution pH and sample consistency also have been found to significantly affect contamination of EP extract blanks.

To determine if solution pH affects the leaching of metals from the extractor, water blanks were run at two additional pH values. These blanks were adjusted to pH 3.5 with 0.1 N nitric acid and to pH 10 with 0.1 ammonium hydroxide. Table 6 contains the results of the inorganic analysis of the blanks for these pH values. The acidic blank contained significantly higher concentrations of Cd, Cr, Cu, Pb, Ni, and Zn than did the alkaline blank.

An additional blank study investigated the effect of sample consistency on extract contamination over the 24-h extraction time. Two types of blanks were run without pH adjustment; one with water and the other with acid-washed sand at a 1:20 solid:solution ratio. Water samples were collected at each point where water was handled before it

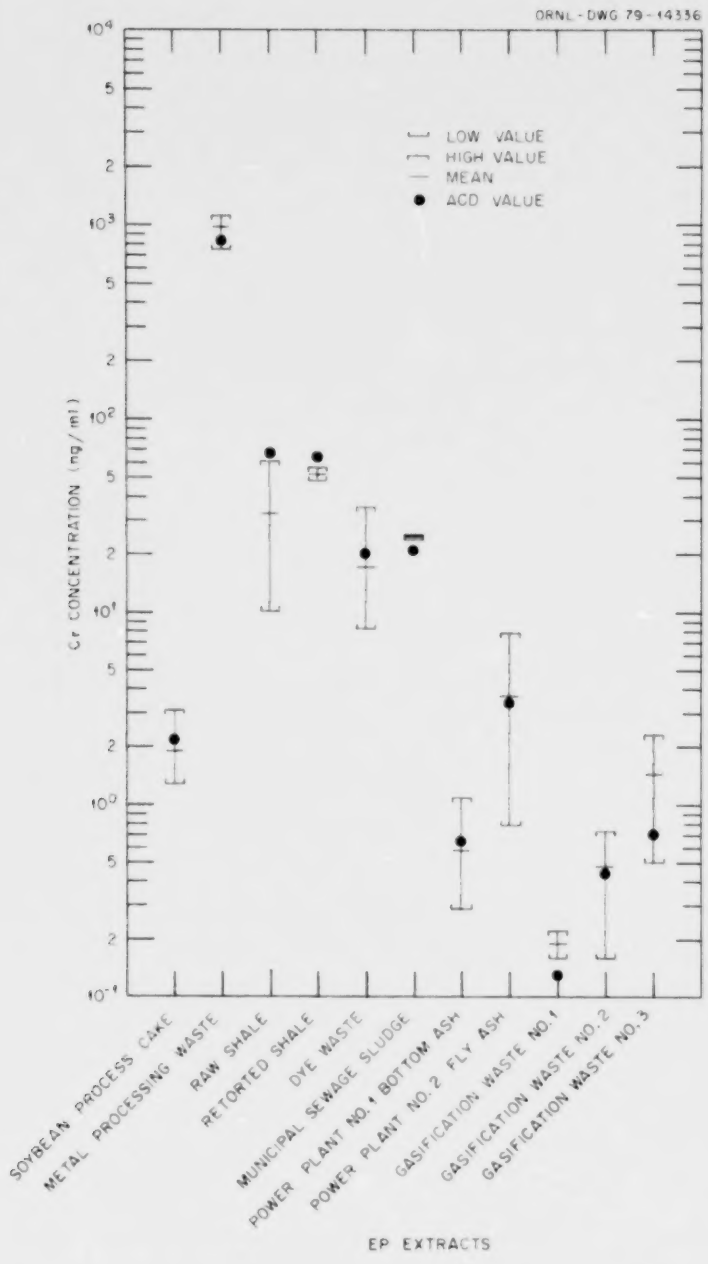

Figure 1. Range and mean chromium concentrations in four separate EP extracts and the Analytical Chemistry Division value after the extracts were combined, for each sample extraction.

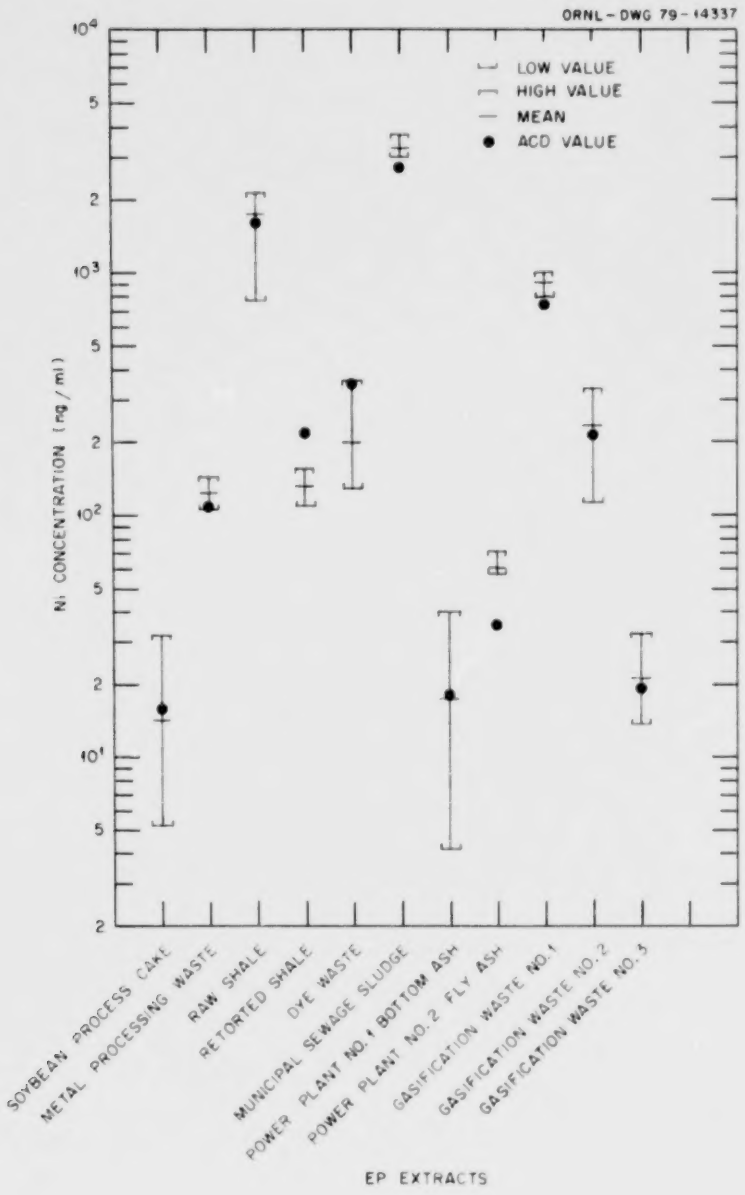

Figure 2. Range and mean nickel concentrations in four separate EP extracts and the Analytical Chemistry Division value after the extracts were combined, for each sample extraction.

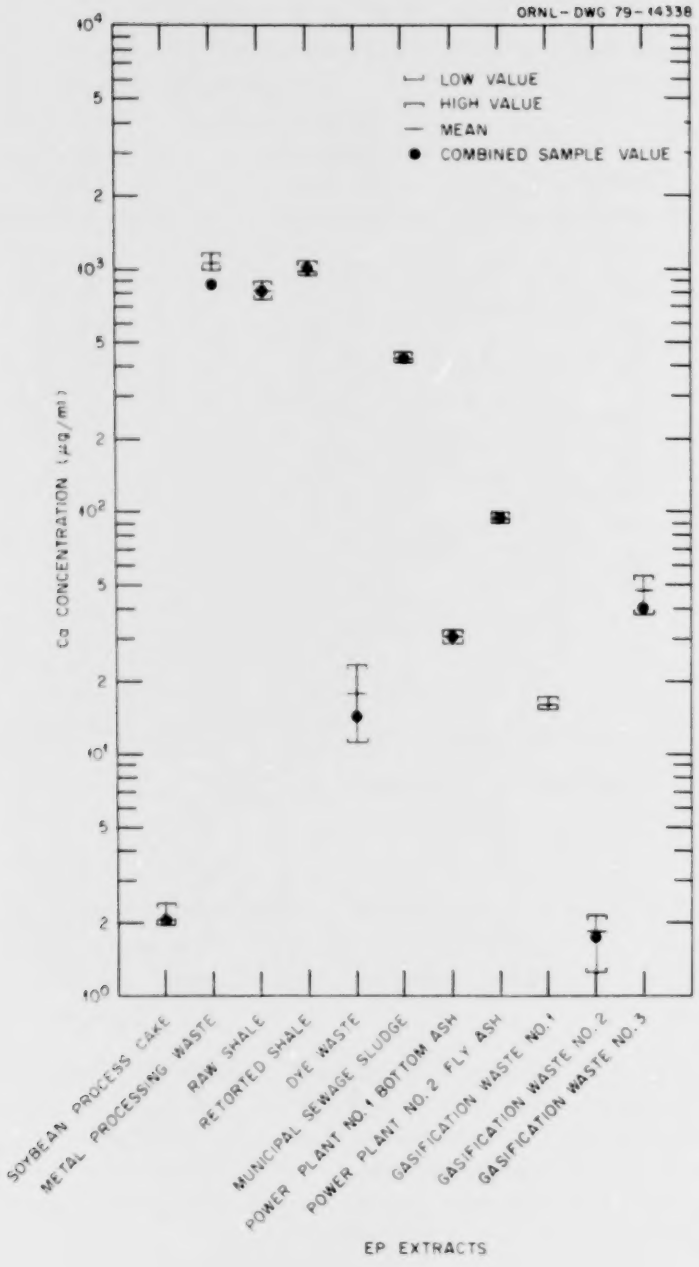

Figure 3. Range and mean calcium concentrations in four separate EP extracts and the concentration value after the extracts were combined, for each sample extraction.

TABLE 6. INORGANIC ANALYSIS OF EP BLANKS AT pH 3.5 AND 10

Element	Concentration (ng/ml) Blank at pH 3.5	Blank at pH 10
Ag	<0.03	<0.03
As	0.23	0.09
Ba	<0.2*	<0.2*
Be	<0.01	<0.01
Cd	0.30	0.019
Cr	37.0	6.1
Cu	52.0	4.0
Hg	0.68	0.42
Ni	107.0	6.4
Pb	0.76	<0.3
Sb	<0.5	<0.5
Se	<5.0	<5.0
Tl	<1.0	<1.0
Zn	45.0	0.57

*Concentration in µg/ml.

reached the Type 316 stainless steel extractor. Additional samples were collected after initial extractor contact, and after 1, 3, 7, and 24 h of stirring. The unfiltered samples from each point were analyzed for Cr and Ni concentrations. Figure 4 contains a plot of the results.

The results of the blank study illustrated in Figure 4 clearly indicate an increased contamination of extracts when a hard granular solid, such as sand, is extracted. Also, over the 24-h extraction time, increasing amounts of extractor contaminants were found. The filtration step in the EP tends to remove a major fraction of the contaminants. Further work is needed to determine if generalizations can be made concerning the removal of abraded metal particles by filtration.

The results of the additional blank studies clearly indicate problems associated with contamination of EP extracts by Type 316 stainless steel extractor materials. Solution pH and sample consistency provide variation relating to this problem. The background level of contamination from the extractor is not constant and does not seem to

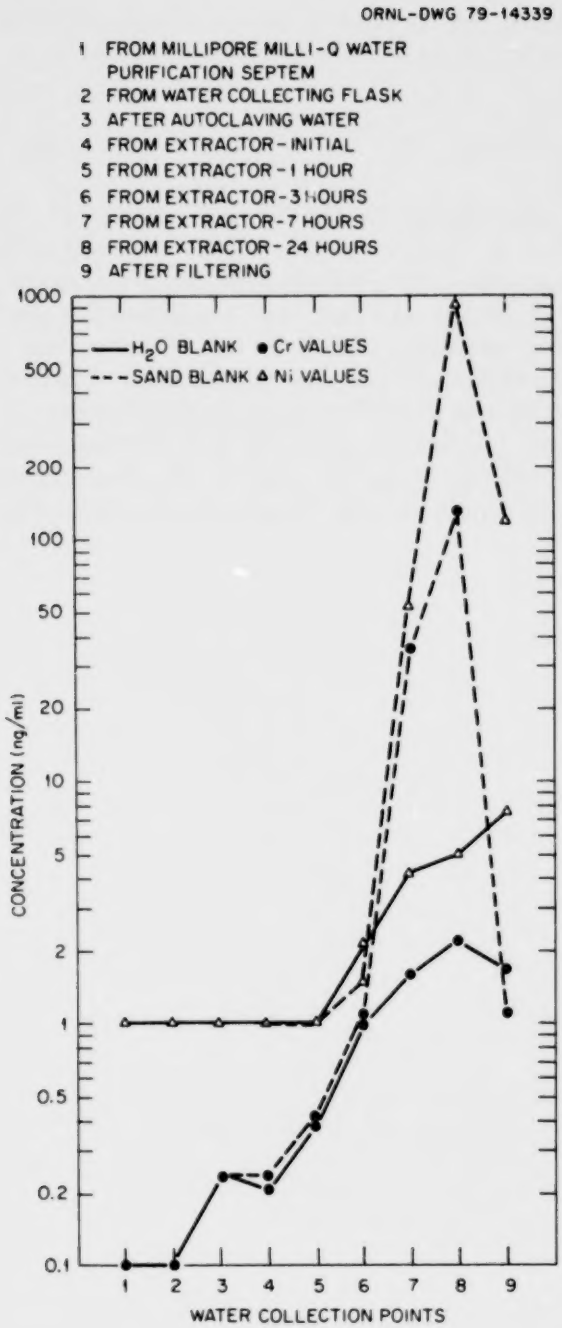

Figure 4. Water and sand blank chromium and nickel concentrations at successive water collection points. Type 316 stainless steel extractors were used with Teflon stirrers.

be predictable. Therefore, we recommend the use of noncontaminating materials such as glass or polytetrafluoroethylene in order to minimize this effect.

FILTRATION OF EP EXTRACTS

Other research activities within ORNL's Environmental Sciences Division have been examining the effect of filtering aqueous solutions containing polyaromatic hydrocarbon (PAH) compounds. Filters studied include those specified in the EP (i.e., Millipore, type HAWP, 0.45-μm pore size, and Nuclepore 0.4-μm pore size membrane filter). Preliminary data indicate a substantial loss of PAH compounds upon filtration. For example, as much as 99% of certain PAH compounds is absorbed by Millipore filters. Due to the preliminary status of the research, the complete data will not be presented here. When the research is complete, data will be presented in the open literature. This type of information, though, may help explain why some expected organic compounds have not been found in Millipore-filtered EP extracts reported in this document.

SECTION 5

CHEMISTRY

INTRODUCTION

The role of the Analytical Chemistry Division in this project was twofold. The primary role was to develop, validate, and apply cost-effective analytical methods for the identification and quantification of selected chemical constituents in the groundwater sample and the 17 EP extracts. Potentially toxic/mutagenic constituents for identification and measurement (Table 7) were selected from the U.S. EPA Primary Drinking Water Standards and the Priority Pollutant List; they included certain trace elements, volatile organics, halogenated organics, and PAHs. The organic constituents were concentrated from the aqueous samples by a macroreticular resin sorption technique. They were fractionated by adsorption column chromatography and identified and measured by gas chromatography (GC). Inorganic constituents were determined directly by atomic absorption spectrometry (AAS).

The secondary role of the Analytical Chemistry Division was to support mutagenesis biotesting in the Biology Division by preparing organic concentrates of the groundwater and EP extracts to concentrate potentially mutagenic constituents into a form more compatible with bioassay. The same macroreticular resin concentration procedure as employed in the analytical studies was used for the bioassay preparations.

In addition to the development and application of analytical methodology, a small scoping effort was also conducted on alternate solid waste extraction procedures suitable for extracting organic materials from the waste.

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODOLOGY

Early analytical studies of aqueous extracts of solid wastes indicated that the organic constituents of interest were present at very low concentrations and that they could be quite complex in nature. Direct analysis was not a viable approach. Thus, a means of efficiently concentrating the organics from the aqueous matrix and of separating them into chemical classes was needed for the successful identification and quantification of the selected organic constituents. A study was also conducted to compare the commonly used AAS with other techniques.

TABLE 7. SPECIFIC COMPOUNDS SELECTED FOR MEASUREMENT IN EP EXTRACTS AND GROUNDWATER SAMPLE

PAH*	Volatile organic	Element	PCB/ pesticide
Acenaphthene	1,1-Dichloroethylene	Ag	Ar-1242
Fluoranthene	Methylene chloride	As	Lindane γ -
Napthalene	trans-1,2-Dichloroethylene	Ba	isomer
Benz(a)anthracene	Acrolein	Be	Methoxychlor
Benzo(a)pyrene	Dichlorobromomethane	Cd	Endrin
Chrysene	Tetrachloroethylene	Cr	Toxaphene
Anthracene	Bromoform	Cu	
Benzo(ghi)perylene	Bis[2-chloroethyl] ether	Hg	
Fluorene	Acrylonitrile	Ni	
Phenanthrene	1,1-Dichloroethane	Pb	
Dibenz(a,c and a,h)anthracenes	Trichloroethylene	Sb	
Pyrene	2-Chloroethyl vinyl ether	Se	
	1,1,2-Trichloroethane	Tl	
	Chlorobromomethane	Zn	
	S-Tetrachloroethane	F	

*Plus 31 other PAHs not included on the Priority Pollutant List.

Preparation of Organic Concentrates

Solvent extraction and macroreticular resin concentration with XAD-2 resin[2,3] were compared for their efficiencies in recovering organics from an aqueous matrix. A mock EP extract was made from 500 ml of triply distilled water acidified with 1.7 ml of glacial acetic acid and spiked with 3×10^5 to 6×10^5 disintegrations/min activity of a ^{14}C-labeled tracer. The pH was then adjusted to 6.8 with $Na3PO_4$, and the conductance was adjusted to 20 mmho/cm with NaCl. The mock EP extract was extracted 5 times with 100-ml portions of organic solvent or was passed through a disposable cartridge containing 4 g of XAD-2 resin (Isolab, Inc., Akron, Ohio) (after reference 2), and the sorbed organics were eluted by the method detailed in Appendix C. Recoveries of the tracers were determined by standard liquid scintillation spectrometric methods. Table 8 shows the compounds tested and the recoveries. Although solvent extraction, particularly with methylene chloride, appears to be the more effective extraction method, it should be noted that the use of methylene chloride in bioassay preparations is questionable. The XAD-2 resin procedure provides a direct 100-fold concentration, and thus the results are not strictly comparable with those for solvent extraction. The results for solvent extraction would have to be adjusted downwards to include the effects of a 100-fold concentration step; therefore there may be little actual difference between the solvent extraction and XAD-2 resin recoveries. Because of the comparable efficiencies of the methods and the

TABLE 8. COMPARISON OF SOLVENT EXTRACTIONS AND XAD-2 RESIN SORPTION FOR RECOVERY OF ORGANICS FROM EP EXTRACTS

Compound	Percent recovery		
	Solvent extraction*		XAD-2 resin†
	Cyclohexane	Methylene chloride	
Hexadecane	100	100	93
Naphthalene	100	100	100
Benzo(a)pyrene	100	100	82
Ar-1254	100	100	95
Stearic acid	100	99	91
Succinic acid	0	0	0
Phenol	3	99	70
Sitosterol	82	100	91
Stearyl alcohol	4	95	90
Indole	41	95	84

*Extraction recovery only.

†Adsorption/desorption and direct 100-fold concentration recovery.

fact that the XAD-2 resin procedure can be applied to as many as 15 samples simultaneously by use of a simple peristaltic pump, we chose the XAD-2 resin concentration procedure as the protocol method for recovery of organics from the EP extracts for both chemical analysis and mutagenic bioassay. Four 500-ml aliquots of each EP extract were subjected to the XAD-2 resin concentration procedure as soon as possible after receipt of the EP extract from the Environmental Sciences Division. EP extracts and concentrates were stored at 4°C in the dark. Two of the four concentrates were chemically analyzed, and two were delivered to the Biology Division for mutagenesis bioassay.

Fractionation and Analysis of Organic Concentrates

The organic concentrates from the EP extracts must be separated into simpler, more well-defined fractions prior to analysis. To accomplish this task in a cost-effective manner, we employed a modified version of our existing adsorption column chromatography procedure[4] for isolation of PAHs from environmental materials. Figure 5 shows the fractionation procedure and the recoveries of radiotracers at various steps. This work was carried out on cyclohexane extracts of mock EP extracts early in the project, but the recoveries beyond the initial solvent extraction should be very similar for organic concentrates prepared with XAD-2 resin. The specific details of the procedure are

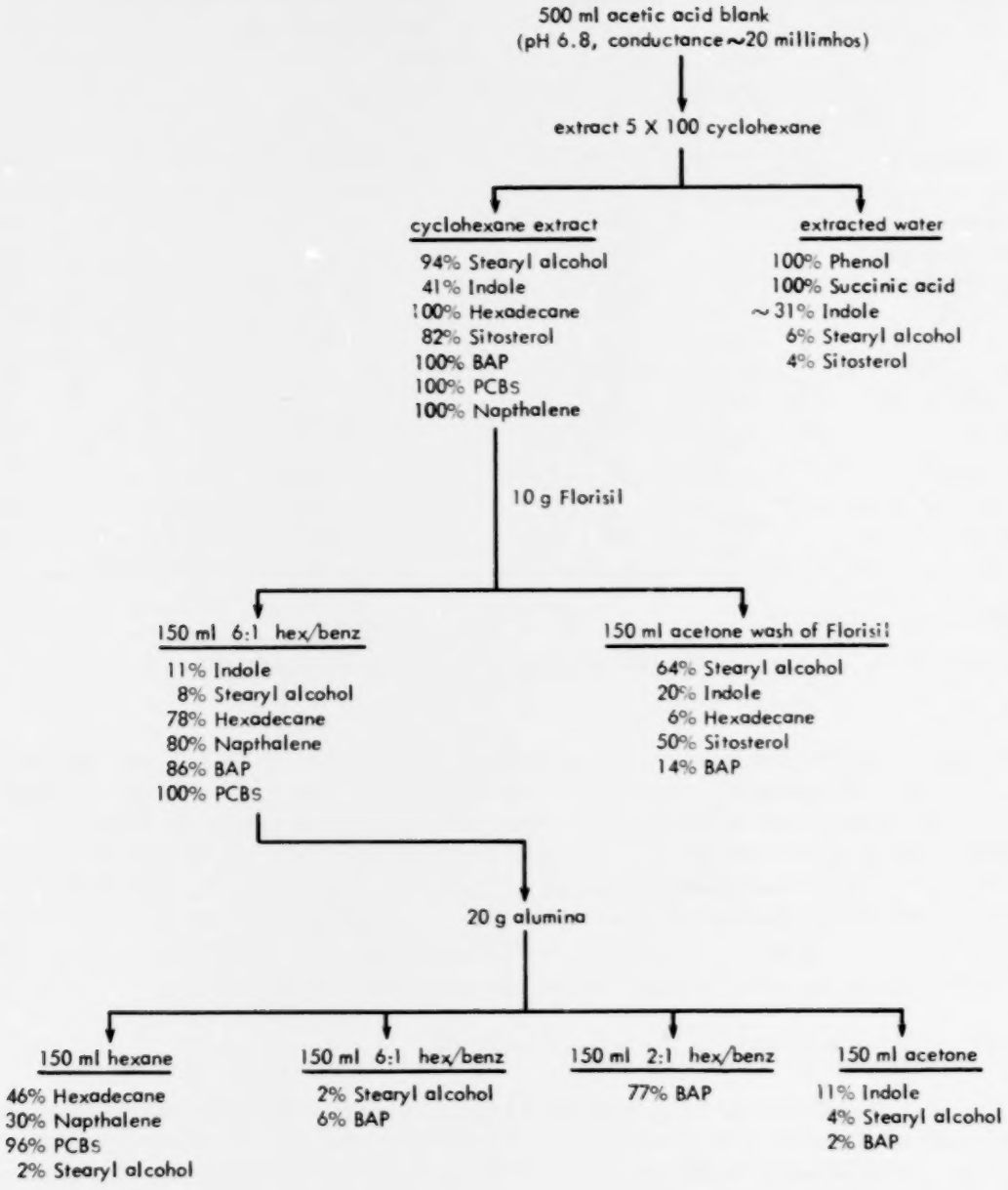

Figure 5. Recoveries of tracers in the fractionation procedure.

included in Appendix C. A preliminary separation of the organic concentrates into nominally nonpolar and polar fractions was accomplished on a Florisil column. The nonpolar fraction was further subdivided into a polychlorinated biphenyl (PCB)/pesticide/monoaromatic/diaromatic/paraffin fraction, a diaromatic fraction (containing the bulk of the diaromatics), a polyaromatic fraction, and a heteroaromatic fraction on an alumina column. Each of these five fractions was concentrated to 1.0 ml and analyzed by GC under the conditions described in Appendix C for the specific constituents chosen and also for any others migrating at the same time. The polar fraction was also screened on the packed Dexsil 400 column for higher-boiling constituents not observed on the Carbowax capillary column, and the diaromatic and polyaromatic fractions were examined with the electron capture detector GC for halogenated and other electronegative constituents.

The parent procedure from which this procedure is derived has been validated[4] for PAH analysis of freshwater sediments and is being evaluated[5] for stream and river water. Two EPA Laboratory Performance Evaluation Standards for PCBs are being analyzed by this procedure.

Analysis of o-Nitroaniline

Tracer studies indicated that the XAD-2 resin procedure is not particularly effective for collection of ionized organics. Thus, the o-nitroaniline content in the arsenic-contaminated groundwater sample was extracted with methylene chloride under conditions more rigorous than those specified in the EPA Level 1 Environmental Assessment Manual[6] and was measured by GC as described in Appendix C. Four extractions were sufficient to remove all solvent-extractable color from the groundwater sample.

Analysis of Volatile Organics

Volatile organic compounds in the EP extracts are present at too dilute concentrations for direct aqueous injection GC. However, they are readily identified and determined by the well-established purge and trap procedure.[7] We adopted the manual version[8] described in Appendix C. The apparatus is shown in Figure 6.

The procedure was evaluated with aqueous standards prepared immediately before analysis with the following toxicants: 1,1-dichloroethane, 1,2-dichloroethane, hexachloroethane, trichloroethane, tetrachloroethane, 2-chloroethyl vinyl ether, chloroform, 1,1-dichloroethylene, 1,2-dichloropropane, 1,3-dichloropropylene, bis[2-chloroisopropyl] ether, bromoform, dichlorobromomethane, trichloroethylene, and dichloromethane. Because of the potentially high losses of these standards from an aqueous sample, the standard was made up at 1 mg/ml in ethylene glycol, and aqueous dilutions were prepared only immediately prior to purging. Thermal desorption and GC analysis of the trapped standards indicated an average precision of ±20%. While the absolute recovery of each toxicant is unknown, recoveries should be highly reproducible for standards and samples treated in the same manner. Sensitivity is approximately 0.1 mg/l when a 1.0-ml sample is analyzed, and greater sensitivity can be achieved by

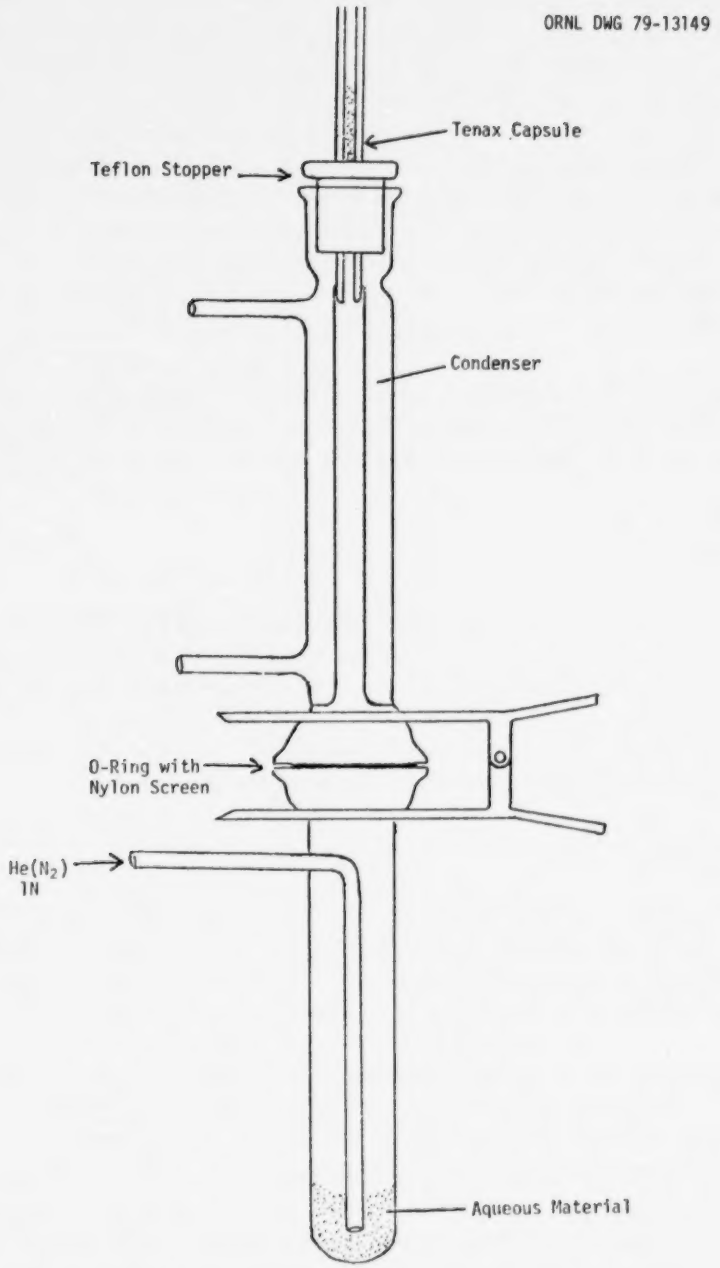

Figure 6. Purge and trap apparatus.

purging a larger aliquot of sample. This procedure also is being tested with EPA Laboratory Performance Evaluation Standards. Aliquots of the EP extracts are taken immediately into bubble-free vials after the last step in the EP procedure and are stored in the dark at -20°C until analysis.

Analysis of Metals

There are several instrumental methods available for determining the content of selected metals (Table 7) in the EP extracts. Because AAS is the most commonly available method, we conducted a comparative study of flameless graphite furnace AAS with five other methods, including spark source mass spectroscopy (with isotope dilution where possible), inductively coupled plasma emission spectrometry, optical emission spectrometry, and instrumental neutron activation analysis. An EP extract of a sewage sludge rather than a standard made up in deionized water was chosen as the test material for comparison of the methods. The sludge should more realistically model some of the interferences to be encountered in the EP extracts of a wide range of solid wastes. The results of quadruplicate analyses by each method (listed in Table 9) indicate that AAS compares well with the other methods, and suggest that it is a valid method for analysis of EP extracts.

Supporting evidence for the validity of our AAS method was obtained in a collaborative study with Mr. Frank Schmidt of the Illinois State EPA. Results for quadruplicate analyses of an EP extract of sewage sludge by AAS in both laboratories are compared in Table 10. Considering the concentration levels, the results are consistent and in generally good agreement. The results for the four footnoted metals did not agree in the first round of the study, but were in agreement in the second round (shown in Table 10). It is likely that inadvertent contamination of the EP extract during packaging for the first shipment caused the disparity in the first round.

Final validation of our AAS method was obtained by analysis of two EPA Laboratory Performance Evaluation Standards for As, Be, Cd, Cr, Cu, Hg, Ni, Pb, and Se. The results were biased slightly low but were acceptably accurate and precise.

Fluoride also was determined in the EP extracts by a specific ion electrode procedure. Because of the lesser importance of fluoride, the procedure did not receive additional validation beyond that in set-up and routine quality control.

Samples of the EP extract for metals analysis were collected in acid-washed glass containers immediately after the last step in the EP. Separate aliquots for Hg determination were preserved with nitric acid and potassium dichromate.

TABLE 9. COMPARISON OF ANALYTICAL METHODS FOR METALS IN EP EXTRACT OF SEWAGE SLUDGE

Element	Average concentration ± S.D. (μg/l) for method*					
	AAS	ID-SSMS	SSMS	ICPS	OES	NAA†
Ag	0.15 ± 0.01		<20	<20		<10
As	30 ± 0		<20			78 ± 3
Be	0.41 ± 0.02				<100	
Cd	1,200 ± 0	1,100 ± 0‡		800 ± 80		1,030 ± 30
Cr	25.0 ± 0.8	<200		≤500		58 ± 6
Cu	700 ± 10	750 ± 70‡		700 ± 70		
Hg	0.026 ± 0.001†					
Ni	3,400 ± 190	4,100 ± 270		3,000 ± 300		
Pb	31 ± 3	<45		<100		
Sb	99.0 ± 1.5		10			41 ± 5
Se	<2		≤10			<20
Tl	12.0 ± 1.4		≤10			
Zn	36,700 ± 678	39,000 ± 2,200		45,000 ± 4,000		55,000 ± 1,500

*AAS, atomic absorption spectrometry; ID-SSMS, isotope dilution-spark source mass spectroscopy; ICPS, inductively coupled plasma emission spectrometry; OES, optical emission spectrometry; NAA, neutron activation analysis. Results are for quadruplicate measurements unless otherwise indicated.

†$\underline{n} = 3$.

‡$\underline{n} = 2$.

ANALYSIS OF EP EXTRACTS

A total of 18 wastes and 12 blanks were subjected to the EPA EP. The results from the chemical analysis of these EP extracts are presented below.

Organic Compound Analysis

A summary of the organic constituent analyses and the estimated limits of detection of each analysis is presented in Table 11. For the volatile organics, a few milliliters of EP extract were collected immediately after the last step in the EP and stored in a freezer until analysis. In spite of these precautions in the collection and preservation of volatile constituents, no volatile organics were detected above a 0.1 mg/l level. This undoubtedly resulted from volatilization losses during the EP 24-h stirring of the wastes in unsealed containers. Use of a sealed container purged with an inert gas through a solid

TABLE 10. COMPARISON OF ORNL AND ILLINOIS STATE EPA ANALYSES OF EP EXTRACT OF SEWAGE SLUDGE*

Element	Average concentration ± S.D. (μg/l) in study by	
	ORNL	Illinois State EPA
Ag†	0.29	<5
As†	6.25 ± 0.22	7
Ba†	<500	400
Be	0.41 ± 0.02	<5
Cd	1,200 ± 0	1,100 ± 0
Cr	25 ± 0.8	38 ± 5
Cu	700 ± 10	830 ± 50
Hg†	0.027‡	< 0.03
Ni	3,400 ± 190	4,000 ± 0
Pb	31 ± 3	41.5 ± 1
Zn	36,700 ± 678	48,500 ± 1,730

*Quadruplicate determinations except where indicated.

†Redetermined in second round of comparative study.

‡$\underline{n} = 2$

adsorbent trap should allow collection and analysis of the volatile organics released from a solid waste during the EP.

Analysis of the arsenic-contaminated groundwater sample did not reveal any detectable volatile organics by the time the analysis was conducted. This result probably was influenced by the handling of the sample prior to our receipt. It arrived in a partially filled plastic jug 3 months after collection, and the conditions of collection, storage, and shipping prior to receipt are unknown. Ample opportunity existed for loss of volatiles.

The analyses for the other protocol organic constituents revealed very little extracted organic material within the limits of sensitivity and the compound classes examined. Nothing was detected which would even approach a "toxic" RCRA classification based on ten times the concentrations listed in the EPA Drinking Water Standards (Table 12). We feel that this is a result of the ionic nature of the EP extractant fluid, in which primarily hydrophilic species would be extracted, leaving the bulk of the essentially hydrophobic species unextracted. The latter would include most of the organic compounds on the Interim Primary Drinking Water Standards and the Priority

TABLE 11. RESULTS OF ORGANIC ANALYSES OF EP EXTRACTS

Extrac	Concentration (ng/l) of sample compound* in extract				
	PAHs (2 µg/l)	Volatile organics (0.1 mg/l)	PCBs† (0.1 ng/l)	Pesticides (0.1–1 ng/l)	Miscellaneous‡
Arsenic-contaminated groundwater	ND§	ND	0.2	ND	
Soybean process cake	ND	ND	0.2	ND	
Metal processing waste	ND	ND	0.2	ND	
Plater's waste	ND	ND	0.2	ND	
Raw shale	ND	ND	0.2	ND	
Retorted shale	ND	ND	0.2	ND	
Dye waste	ND	ND	0.4	ND	paraffins
Textile waste	ND	ND	0.3	ND	paraffins
Municipal sewage sludge	ND	ND	0.4	ND	paraffins†
Power plant No. 1 fly ash	ND	ND	0.2	ND	
Power plant No. 1 bottom ash	ND	ND	0.2	ND	
Power plant No. 1 scrubber sludge	ND	ND	0.2	ND	
Power plant No. 1 treated scrubber sludge	ND	ND	0.3	ND	paraffins
Power plant No. 2 fly ash	ND	ND	0.4	ND	paraffins
Fluidized bed residue	ND	ND	0.2	ND	
Gasification waste No. 1	ND	ND	0.2	ND	
Gasification waste No. 2	ND	ND	0.2	ND	
Gasification waste No. 3	ND	ND	0.2	ND	
Blanks 1–5	ND	ND	1.0	ND	

*Detection limits for 500-ml sample are shown in parentheses.

†Identification of Ar-1242 in EP extracts is tentative, based on GC retention only.

‡See Table 15.

§ND, not detected.

TABLE 12. SELECTED ORGANIC AND INORGANIC CONSTITUENTS IN THE EPA INTERIM PRIMARY AND PROPOSED SECONDARY DRINKING WATER STANDARDS*

Standard	Constituent	Maximum concentration (μg/l)
Interim primary	Ag	50
	As	50
	Ba	1,000
	Cd	10
	Cr	50
	F	†
	Hg	2
	Pb	50
	Se	10
	Endrin	2
	Lindane	4
	Methoxychlor	100
	Toxaphene	5
Secondary	Cu	†
	Zn	†

*Source: Federal Register, 43(232), 59019 (Dec. 18, 1978).

†None set.

Pollutants List. In addition, there is some evidence (e.g., reference 9) that membrane filtration, such as that used in the RCRA protocol EP, can effectively adsorb organics from aqueous solution.

However, there was some indication of organics in the EP extracts. All samples contained PCBs at levels below 1 ng/l, most of which probably originated from the system blank and not the waste sample itself. Figure 7 shows electron capture detection GC of an Aroclor (Ar)-1242 standard and the PCB/pesticide fraction from the EP extract of the fluidized bed residue. The GC profiles correspond nearly identically, and the PCB concentration in the EP extract is estimated to be approximately 0.2 ng/l. Identification was based on the GC profile; PCB concentrations were too dilute for successful mass spectroscopic identification.

Some nonprotocol constituents were identified and measured in the samples. A few EP extracts were found to contain n-paraffins, as reported in Table 13; these were limited mainly to n-$C_{20}H_{42}$ through n-$C_{25}H_{52}$, although traces of both heavier and lighter homologs were present. The paraffins represent the most concentrated organic compounds identified in the EP extracts, but their concentrations do not appear to reflect the expected organic compound content of the wastes. The observation that the EP

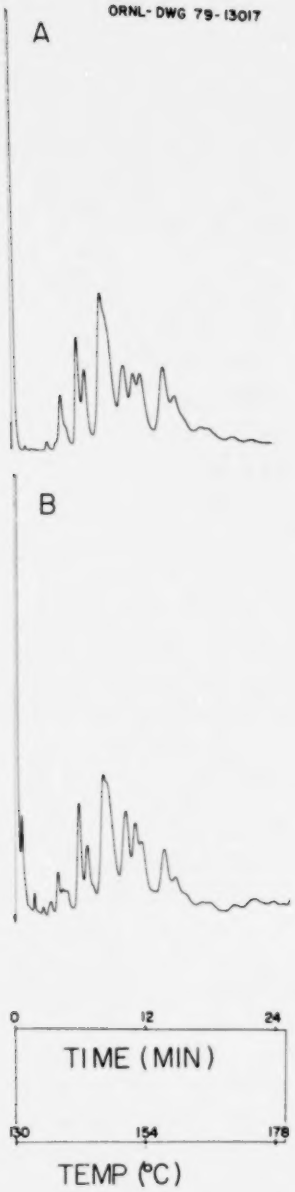

Figure 7. Gas chromatogram of (A) Ar-1242 standard equivalent to 0.2 ng/l and (B) PCB/pesticide fraction from EP extract of fluidized bed residue.

TABLE 13. CONCENTRATIONS OF n-PARAFFINS IN EP EXTRACTS

Extract	Concentration of n-paraffin (µg/l)										
	$C_{17}H_{36}$	$C_{18}H_{38}$	$C_{19}H_{40}$	$C_{20}H_{42}$	$C_{21}H_{44}$	$C_{22}H_{46}$	$C_{23}H_{48}$	$C_{24}H_{50}$	$C_{25}H_{52}$	$C_{26}H_{54}$	Total
Dye waste	—	—	1.6	7.2	14	23	28	16	6.8	1.9	98.5
Power plant No. 1 treated scrubber sludge	—	—	—	3.3	6.1	13	13	7.6	3.3	2.4	48.7
Power plant No. 2 fly ash	—	2.6	3.3	8.2	18	31	38	23	10	4	138.1
Textile waste	—	—	1	5	12	15	18	10	4	2	67.0
Municipal sewage sludge	2	2	2	6	12	26	26	16	10	8	110.0

extract of fly ash contained higher concentrations of paraffins than those of textile waste or sewage sludge, and the similarities in the distributions of the various paraffins, suggest a common source. However, none of the EP extract blanks contained paraffins.

A specific determination was made of the o-nitroaniline content of the arsenic-contaminated groundwater. Because we were unsure of its recovery from water in the XAD-2 resin procedure, we used a more efficient methylene chloride EP. GC analysis indicated an o-nitroaniline concentration of 320 mg/l. No other organic compounds were detected in the analysis, supporting the results obtained with the XAD-2 organic concentrate of the groundwater sample. This result for o-nitroaniline groundwater concentration far exceeds the maximum permissible EP extract concentration level (30 mg/l) calculated from rat oral LD_{50} data obtained from the NIOSH Registery,[10] and it is the only organic compound data which could result in a "toxic" RCRA classification, based on the proposed delisting criteria as a toxicity characteristic.

Inorganic Analysis

The results for the metals analyses of the arsenic-contaminated groundwater and the EP extracts of the 17 wastes are presented in Table 14. These data are rounded off to the nearest μg/l except those for Hg (lower Standard) to facilitate comparison with RCRA regulations. In comparison with the results of the organic analyses, these data suggest that the EP extracts are predominantly ionic in character, as would be expected of compounds extracted by an aqueous acidic extraction fluid. By the criterion of a maximum permissible metal concentration in the EP extracts of 10 times the EPA Interim Primary Drinking Water Standards (Table 12), three wastes (metal processing waste, plater's waste, and municipal sewage sludge) plus the arsenic-contaminated groundwater sample would be labeled by RCRA as "toxic" because of their extractable As, Cd, or Cr contents, and one (power plant No. 1 fly ash) would be a borderline case due to its extractable Cd content. However, a power plant fly ash sample from a second source, as well as bottom ash, scrubber sludge, and treated scrubber sludge, contained far less extractable Cd. None of the other energy-associated wastes (e.g., gasification wastes, fluidized bed residue, shale) would receive a toxic classification by these criteria.

If EPA decided to expand the proposed toxicity characteristic (43 FR 58956) to include the EPA Secondary Drinking Water Standards, then the dye waste also would be classified as "toxic" because of its extractable Cu content. The extractable Zn and Cu content of the plater's waste and the Zn content of municipal sewage sludge would provide additional evidence for their "toxic" classifications. The extractable fluoride content of the municipal sewage sludge would be a borderline classification. A measurement of cyanide in the plater's waste EP extract revealed a 156 mg/l concentration. From these results, the plater's waste and the waste contributing to the arsenic-contaminated groundwater sample would be potentially the most "toxic" waste materials of the 18 examined.

TABLE 14. RESULTS OF METALS ANALYSES OF EP EXTRACTS*

Extract	Concentration of element (μg/l)														
	Ag	As	Ba	Be	Cd	Cr	Cu	Hg	Ni	Pb	Sb	Se†	Tl	Zn	F
Arsenic-contaminated groundwater	5	412,000	<500	2	485	2	10	0.5	935	117	297	11	7,720	251	ND‡
Soybean process waste	<1	2	<500	<1	<1	2	69	0.1	16	3	<2	<1	<1	110	<100
Metal processing waste	<1	<1	<500	1	3.0	823	12	0.1	109	2.0	26	3	<1	75	2,000
Plater's waste	34	1	<500	1	1,850	3,110	165,000	0.9	4,410	38	13	<1	4	285,000	3,300
Raw shale	<1	<1	3,700	3	1	67	6	0.2	1,610	3	2	1	<1	295	3,700
Retorted shale	1	9	800	5	<1	64	11	0.1	221	1	9	6	<1	<10	12,000
Dye waste	2	1	<500	<1	3.0	20	326,000	0.4	355	38	4.0	<4	<1	260	<100
Textile waste	<1	1	<500	1	<1	1	13	0.1	131	1	23	<3	<1	85	<100
Municipal sewage sludge	<1	6	<500	<1	524	21	413	0.4	2,757	9	25	3	3	140,500	400
Power plant No. 1 fly ash	<1	1	<500	7	100	5	46	<0.1	662	2	36	<1	21	1,550	8,000
Power plant No. 1 bottom ash	<1	2	<500	<1	1	1	14	0.1	18	<1	<2	2	<1	35	<100
Power plant No. 1 scrubber sludge	1	47	<500	4	2	10	20	0.1	140	2	29	30	12	235	3,000
Power plant No. 1 treated scrubber sludge	<1	54	<500	2	4	13	49	0.1	6	3	3.0	43	39	810	5,900
Power plant No. 2 fly ash	<1	400	<500	1	9	3	102	0.1§	35	<1	28	ND	10	515	1,300
Fluidized bed residue	8	1	<500	4	<1	12	22	0.2	<5	<1	<1	<3	<1	1	400
Gasification waste No. 1	<1	1	<500	<1	3	<1	1	0.2	758	2	<2	<1	<1	3,850	<100
Gasification waste No. 2	<1	<1	<500	<1	1	<1	4	<0.1	219	<1	<2	2	<1	10	<100
Gasification waste No. 3	<1	<1	<500	<1	2	1	20	<0.1	19	1	<1	1	<1	770	<100

*Average of duplicate measurements except as noted.

†Single determination except for groundwater and power plant No. 1 fly ash.

‡ND, not determined.

§Single determination.

The increasing concentration trend for the metals Be, Cd, Cu, Ni, Pb, Sb, Tl, and Zn in bottom ash < scrubber sludge < fly ash is interesting to note. The result is consistent with the preferential accumulation of many of these elements in fly ash versus bottom ash.[11] However, other volatile metals, such as Hg, do not show the expected concentration trends in the EP extracts.

A white precipitate formed in the EP extract of power plant No. 1 scrubber sludge shortly after it was generated. An optical emission spectroscopic survey analysis indicated that the major element was Ca, as would be expected from the limestone employed in the scrubber. Approximately 0.1 mg/g of Mg and B also were detected in the precipitate.

Although the RCRA protocol does not specify EP extract blanks, extraction blanks were generated and analyzed to check the background levels of inorganic constituents. Table 15 shows the analytical results for multiple blanks generated by the three types of apparatus: A Plexiglas container with steel stirrer, a stainless steel container with stainless steel stirrer blades, and a stainless steel container with Teflon stirrer blades. There appear to be small differences among the blanks generated in the three apparatus; notably between the Plexiglas and stainless steel apparatus. Because the former was operated uncovered, the high result for one Zn determination may have been caused by airborne contamination of the extraction vessel. The Plexiglas blank is appropriate for the EP extracts of power plant No. 1 fly ash, bottom ash, and scrubber sludge; gasification wastes No. 1–3; soybean process cake; metal processing waste; and plater's waste. The stainless steel apparatus with the Teflon blades was used for the fluidized bed residue, municipal sewage sludge, and power plant No. 2 fly ash EP extracts. The stainless steel apparatus with the stainless steel blades was used for all the other EP extracts. Although the magnitudes of the blanks are small in comparison with the maximum permissible concentrations of metals, they are significant with respect to the metals concentrations in some of the EP extracts, and they may decide a borderline case such as the Cd level in the power plant No. 1 fly ash EP extract. Thus we feel that appropriate EP extract blanks should be included in the RCRA protocol to aid in classification of borderline cases.

ALTERNATE EXTRACTION PROCEDURES

The apparent failure of the EP protocol to produce extracts whose organic contents mirror that expected to occur in a disposal environment has spurred the search for a more meaningful extraction regimen with respect to organic pollutants. Several factors were considered. The procedure should be (1) somewhat relevant to the actual (or supposed) environmental extraction of these materials; (2) directly applicable to solid wastes without physical modification of the waste (and, we hope, without regard for water content); (3) amenable to routine analysis, i.e., rapid, facile, and quantitatively accurate.

TABLE 15. METALS ANALYSES OF EP EXTRACT BLANKS IN THREE TYPES OF APPARATUS

Element	Average concentration ± S.D. (μg/l) for apparatus		
	Plexiglas*	Stainless steel†	Stainless steel‡
Ag	<1	<1	<1
As	<1	<1	<1
Ba	<500	<500	<500
Be	<1	<1	<1
Cd	1 ± 2	<1	<1
Cr	1 ± 1	2 ± 2	3 ± 2
Cu	4 ± 4	24 ± 14	14 ± 16
Hg	0.2 ± 0.1	0.1 ± 0.04	0.10 ± 0.1
Ni	13 ± 13	38 ± 9	25 ± 25
Pb	1 ± 1	<1	<1
Sb	<2	<1	<1
Se	<0.7	<3	<3
Tl	<1	<1	<1
Zn	282 ± 480	24 ± 15	38 ± 10
F	<100	<100	<100

* $\underline{n} = 4$.

† $\underline{n} = 5$; stainless steel stirrer.

‡ $\underline{n} = 3$; Teflon stirrer.

The traditional method for the extraction of organics from solids is Soxhlet extraction with a volatile organic solvent. This method fails to have any environmental relevance, however, and moisture content can be a key parameter unless a water-miscible organic solvent is employed. Furthermore, the physical state of certain solids can be artificially altered by the direct application of organic solvents.

Clearly some form of aqueous treatment is necessary to more closely model environmental extractions. Steam distillation was considered as one possible model for the removal of organic pollutants from solid wastes.

Because the extraction is from an aqueous system, the moisture content of the waste is not a controlling factor, and quantitation can be performed on a volumetric basis. The physical state of the solid is also irrelevant, transfer to the flask being the only limitation. The procedure requires no technical bench time after the extraction begins.

The apparatus used in steam distillation (Figure 8) is commercially available (Kontes Glass Co., Vineland, N. J., Part No. K523010). Briefly, the solid waste (100 g) is added to a flask containing 250 ml of triply distilled water (represented by the dark-colored fluid in Figure 8). The water is brought to a boil to accomplish steam distillation. Concurrently, an immiscible organic solvent (represented by the light-colored fluid in Figure 8) is distilled on the opposite side of the vessel. The steam and organic vapors co-condense, and the two phases are separated and returned to their respective vessels. Thus, an extraction is carried out in which the organic solvent does not come into contact with the solid waste.

Pilot studies with this procedure have been promising. The recovery of benzanthracene from water is virtually 100%, whereas higher-boiling pollutants such as benzo(a)pyrene are only partially recovered. Furthermore, the recovery depends on the nature of the solid waste.

Recovery of the benzanthracene tracer from the metal processing waste was 60%, whereas recovery from the soybean process cake was essentially 0. Recovery is a measure of the adsorptivity of the solid, and adsorptivity probably affects the release of organics into the environment from a landfill disposal site.

Two of the solid waste samples that were subjected to the EP and subsequent biological, analytical, and ecological testing were chosen for evaluation based on this technique; the two samples, metal processing waste and soybean process cake, were selected based on their substantial physical differences, one being a clay and the other resembling activated charcoal. Neither of these materials showed significant biological activity when tested by the proposed RCRA procedure. ^{14}C-labeled benz(a)anthracene was used for recovery measurements. GC on an OV-101 glass capillary column was used for preliminary screening of the total extract as well as for analysis of the fractions after isolation.

GC analysis of the two steam distillation extracts followed the pattern expected from the tracer recoveries. The metal processing waste was considerably more complex in organic content than the soybean process cake (Figures 9 and 10). The extract of the former, representing 0.2% by weight of the solid, was fractionated to obtain an isolate containing PAHs. Fluoranthene, pyrene, benz(a)anthracene, chrysene, and benzo(b)fluoranthene were tentatively identified by their GC retention times. Again, no PAHs were found in the EP extract.

The steam distillate extracts were also subjected to mutagenicity testing in the Ames/Salmonella system (see Sect. 8). The metal processing waste again showed significant activity, but the soybean process cake was inactive. Thus, this extraction procedure offers a substantial improvement over the EP, at least with respect to recovery of organic pollutants. An extraction procedure such as this, effective for organic compounds, should be incorporated into the RCRA protocol.

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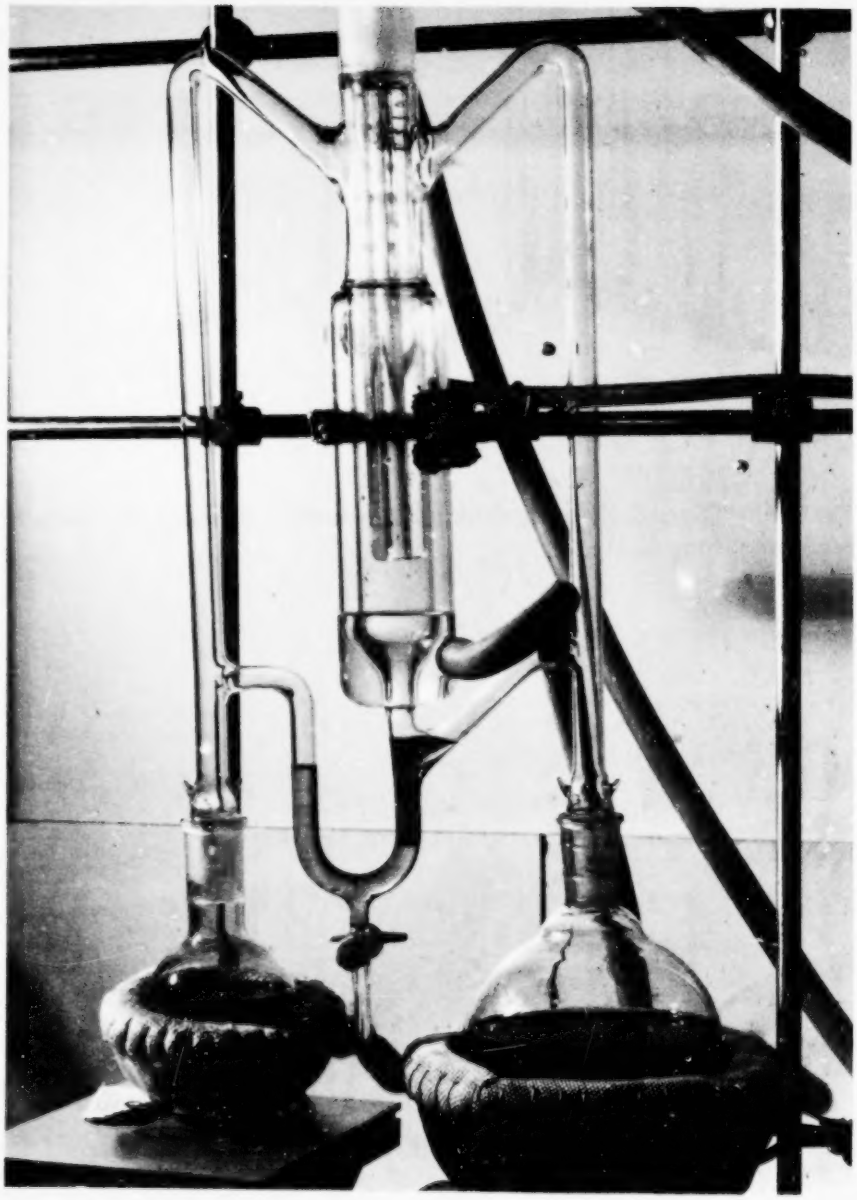

Figure 8. Steam distillation apparatus for solid waste extraction.

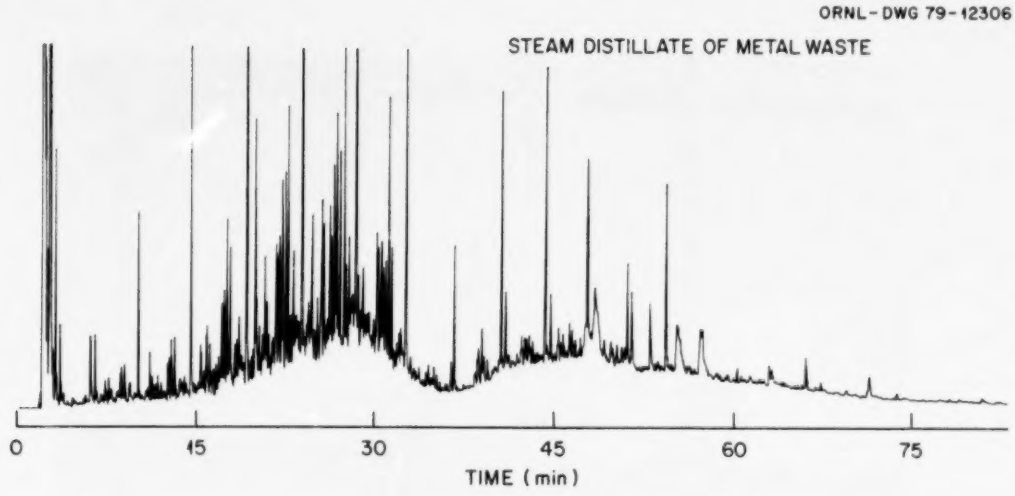

Figure 9. Glass capillary column GC separation of steam distillate from metal processing waste.

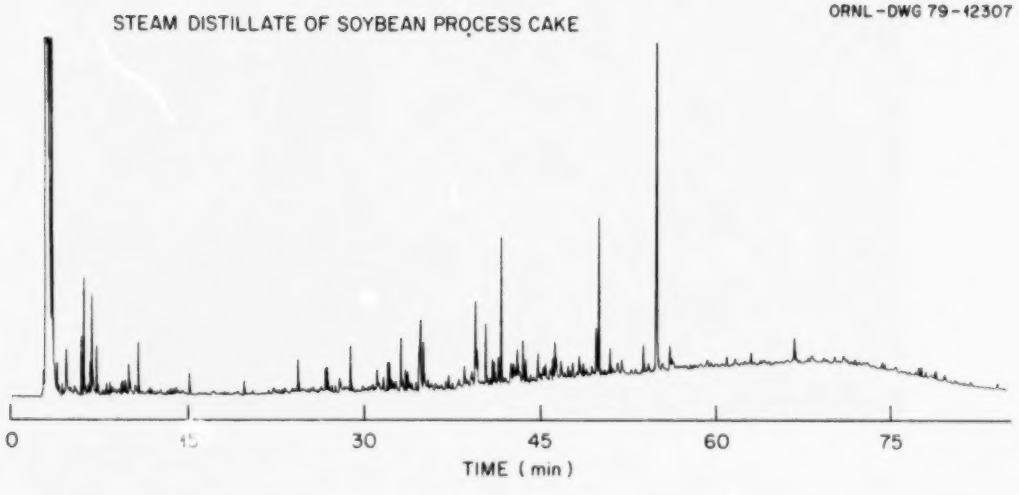

Figure 10. Glass capillary column GC separation of steam distillate from soybean process cake.

CONCLUSIONS AND RECOMMENDATIONS

The overriding conclusion from the chemical analyses is that the current EP protocol is ineffective in extracting organic compounds from solid wastes. The ionic nature of the extractant fluid in the current EP, the open system extraction, and possibly the membrane filtration step work against extraction and recovery of organics. As would be expected, the EP extracts generated by the current EP are predominantly ionic in character, and only the dissolved metals analyses are useful for determination of the "toxic"/"nontoxic" classification of the solid waste. It probably is not necessary to conduct organic compound analyses of the extracts generated by the current EP, although a relatively inexpensive screening procedure such as that for total organic carbon might be useful for setting priorities for organic analyses if acetic acid was not included in the EP.

Alternate extraction procedures may be necessary for assessment of extractable organic compounds. One possible candidate is the continuous steam distillation/organic solvent extraction method examined in our pilot study.

Research should be conducted into the physical/chemical properties of wastes which dictate availability of organic/inorganic constituents and on means of cost-effective determination of these properties in the wastes. When available, authentic landfill leachates should be compared with extracts prepared in the laboratory to assess the environmental relevance of an extraction procedure. The ideal extraction procedure would reproduce the composition of an actual leachate. Direct analysis of the solid waste also should be considered.

SECTION 6

AQUATIC TOXICITY

The purposes of the aquatic toxicity tests were: (1) to evaluate a short-term screening test for its suitability for determining if an EP extract poses a hazard to the aquatic ecosystem under conditions described in EPA's draft RCRA 83001 Toxicity Background Document, and (2) to determine the toxicity of the arsenic-contaminated groundwater and 17 waste extracts to aquatic organisms. Lethal or acute (short-term exposure) tests and chronic (long-term) tests, which determined sublethal effects on reproduction, were used. The test organism was the cladoceran, Daphia magna, which is used as a standard organism in toxicity testing because of its sensitivity to many chemicals. From the results of the acute tests, the concentration of the extract that was lethal to D. magna during an exposure period of 48 h was estimated. From the chronic test results, it was determined if continuous exposure to extract dilutions of 1:100 and 1:1000 impaired reproduction of D. magna. The chronic tests did not, therefore, establish a dose-response relationship, such as an LC_{50}, for the extract but only determined if the extract was toxic at the concentrations tested. The 1:1000 dilution factor was proposed by EPA as being a reasonable dilution factor for protection of aquatic organisms from chronic exposure to leachates from landfill waste.[1]

The test procedures are described in Appendix D. The five criteria used to assess the effects on D. magna reproduction in the chronic tests were survival time, day of first brood release, number of broods per adult, number of young per brood, and total number of young produced per adult. From the results of the chronic exposure tests, the most consistently sensitive criteria were determined for evaluation of toxic effects. The statistical tests used to compare the sensitivities of the above criteria were analysis of variance combined with Duncan's Multiple Range Test to identify significant differences among individual treatment means. A type I error (α) of 0.05 was selected as the level of significance.

RESULTS

The results of the acute toxicity tests are shown in Table 16, and those of the chronic tests are presented in Table 17. A summary of the results for both types of tests and their significance is given in Table 18. The second acute toxicity test was done 28 days after the first one to determine if the toxicity of the test material had changed

TABLE 16. ACUTE TOXICITY OF WASTE EXTRACTS TO D. MAGNA

Extract	48-h LC_{50}* (95% fiducial limits)	
	First test	Second test
Arsenic-contaminated groundwater	2.2†	1.7†
Soybean process cake	‡	§
Metal processing waste	28.0†	39.0†
Plater's waste	0.005	not done
Raw shale	53.9 (47.1–62.3)	64.4 (56.1–77.6)
Retorted shale	70.0 (30.0–>100)	39.4 (30.7–51.4)
Dye waste	0.0005†	not done
Textile waste	19.2 (16.8–22.5)	35.5†
Municipal sewage sludge	7.5†	3.0†
Power plant No. 1 fly ash	90 (71–>100)	69†
Power plant No. 1 bottom ash	94†	60†
Power plant No. 1 scrubber sludge	85 (51–>100)	60†
Power plant No. 1 treated scrubber sludge	81.1 (72.8–91.0)	95.6 (85.2–>100)
Power plant No. 2 fly ash	15.2†	11.6†
Fluidized bed residue	12.6 (10.8–15.0)	25.0
Gasification waste No. 1	§	§
Gasification waste No. 2	100.0†	‡
Gasification waste No. 3	70.0 (29.4–>100)	46.0†

*Estimated percent concentration of the extract that killed or immobilized 50% of the test organisms in 48 h.

†Approximate value; 95% fiducial limits could not be calculated.

‡No significant mortality of test organisms in 48 h in 100% of the extract.

§Less than 50% of the test organisms were killed or immobilized in 100% of the extract in 48 h.

during that time, which was the duration of the chronic toxicity tests. Extracts were stored at 4°C during the 28 days.

A material was considered to have caused a toxic effect when there was a statistically significant reduction ($\alpha = 0.05$) in the mean number of young produced by each organism at either test dilution compared with the number of young produced by control organisms exposed to the dilution water alone and dilution water with acetic or hydrochloric acid. Of the above-mentioned five criteria used to assess toxicity, the mean number of young per adult and the number of young per brood were the most consistently sensitive; the first was chosen for convenience as the toxicity criterion.

TABLE 17. CHRONIC TOXICITY OF WASTE EXTRACTS TO D. MAGNA*

Extract and concentration	Life length during study (days)	Day of first brood release	No. of broods/adult	No. of young/adult	No. of young/brood
Arsenic-contaminated groundwater					
Control	$27.2^{a} \pm 1.8$	$11.3^{a} \pm 1.0$	$5.2^{a} \pm 0.8$	$90.3^{a} \pm 15.4$	$17.8^{a} \pm 4.1$
1:1000	$26.1^{a} \pm 4.2$	$12.5^{a} \pm 1.6$	$4.8^{a} \pm 1.1$	$79.5^{a} \pm 24.3$	$16.5^{a} \pm 3.4$
1:100	$28.0^{a} \pm 0.0$	$13.7^{b} \pm 1.0$	$5.0^{a} \pm 0.8$	$11.2^{b} \pm 10.8$	$2.1^{b} \pm 1.7$
Soybean process cake					
Control	$27.4^{c} \pm 1.3$	$11.3^{c} \pm 1.0$	$6.6^{c} \pm 1.0$	$99.2^{d} \pm 16.3$	$15.1^{d} \pm 1.9$
Acetic acid control†	$27.5^{c} \pm 1.6$	$11.0^{c} \pm 0.0$	$6.5^{c} \pm 0.7$	$118.5^{c,d} \pm 23.2$	$18.2^{c,d} \pm 2.6$
1:1000	$27.7^{c} \pm 1.0$	$11.0^{c} \pm 0.0$	$6.8^{c} \pm 1.0$	$111.8^{c,d} \pm 31.4$	$16.7^{c,d} \pm 4.9$
1:100	$27.5^{c} \pm 1.6$	$11.0^{c} \pm 0.0$	$6.5^{c} \pm 1.2$	$131.7^{c} \pm 23.7$	$20.5^{c} \pm 3.5$
Metal processing waste					
Control	$27.3^{e} \pm 2.2$	$9.2^{e} \pm 0.6$	$6.9^{e} \pm 1.1$	$115.9^{f} \pm 17.8$	$17.0^{f} \pm 1.7$
Acetic acid control†	$28.0^{e} \pm 0.0$	$10.0^{e} \pm 2.1$	$7.0^{e} \pm 0.7$	$155.7^{e} \pm 29.3$	$22.3^{e} \pm 4.0$
1:1000	$28.0^{e} \pm 0.0$	$9.2^{e} \pm 0.6$	$6.9^{e} \pm 0.6$	$118.6^{f} \pm 16.8$	$17.2^{f} \pm 2.2$
1:100	$28.0^{e} \pm 0.0$	$9.0^{e} \pm 0.0$	$7.2^{e} \pm 0.4$	$155.8^{e} \pm 24.9$	$22.1^{e} \pm 3.7$
Raw shale					
Control	$29.0^{g} \pm 0.0$	$11.8^{g} \pm 1.5$	$6.2^{g} \pm 0.6$	$102.8^{h,i} \pm 12.8$	$16.6^{h,i} \pm 1.8$
Acetic acid control†	$28.8^{g} \pm 0.6$	$12.4^{g} \pm 1.3$	$5.9^{g} \pm 0.7$	$111.1^{h} \pm 28.0$	$18.8^{h} \pm 4.2$
1:1000	$29.0^{g} \pm 0.0$	$12.1^{g} \pm 1.4$	$5.7^{g} \pm 0.5$	$89.9^{i} \pm 20.1$	$15.3^{i} \pm 3.3$
1:100	$28.4^{g} \pm 1.3$	$11.5^{g} \pm 1.6$	$6.2^{g} \pm 0.4$	$145.3^{g} \pm 23.3$	$23.5^{h} \pm 4.2$

(continued)

(Table 17 continued)

Extract and concentration	Life length during study (days)	Day of first brood release	No. of broods/adult	No. of young/adult	No. of young/brood
Retorted shale					
Control	27.7[j] ± 0.9	9.8[j] ± 2.3	5.3[j] ± 1.2	84.1[j] ± 28.6	15.8[j] ± 3.8
Acetic acid control[†]	28.8[j] ± 0.0	10.2[j] ± 2.1	5.2[j] ± 0.9	67.6[j] ± 18.1	13.0[j,k] ± 2.5
1:1000	27.0[j] ± 3.2	8.4[j] ± 1.0	4.9[j] ± 1.0	78.0[j] ± 23.3	16.0[j] ± 3.9
1:100	28.0[j] ± 0.0	9.9[j] ± 1.7	5.6[j] ± 0.7	64.6[j] ± 18.1	11.5[k] ± 2.7
Textile waste					
Control	26.0[ℓ] ± 5.8	9.5[ℓ] ± 2.2	6.1[ℓ] ± 2.0	57.3[m] ± 20.9	9.3[m] ± 2.6
Acetic acid control[†]	29.0[ℓ] ± 0.0	9.4[ℓ] ± 1.0	7.5[ℓ] ± 1.5	119.0[ℓ] ± 29.5	15.8[ℓ] ± 2.1
1:1000	27.6[ℓ] ± 4.4	9.8[ℓ] ± 2.9	6.4[ℓ] ± 2.2	55.7[m] ± 20.4	8.6[m] ± 1.4
1:100	27.1[ℓ] ± 4.2	9.0[ℓ] ± 1.7	6.5[ℓ] ± 2.2	83.5[n] ± 40.0	12.2[n] ± 3.4
Municipal sewage sludge					
Control	27.2[o] ± 2.5	11.0[o] ± 10.0	6.5[o] ± 1.0	150.0[o] ± 44.3	23.0[o] ± 5.5
Acetic acid control[†]	26.3[o] ± 5.4	10.3[o] ± 3.7	6.0[o] ± 2.2	131.3[o] ± 66.0	22.1[o] ± 10.0
1:1000	24.1[o] ± 6.6	11.5[o] ± 4.4	3.0[p] ± 1.9	15.1[p] ± 12.3	4.6[p] ± 2.8
1:100	15.6[p] ± 4.8	—[p,‡]	0.1[q] ± 0.3[‡,§]	0.0[p,§]	0.0[p,§]
Power plant No. 1 fly ash					
Control	25.8[r] ± 4.7	10.8[r] ± 0.6	5.5[r] ± 1.5	106.1[r] ± 36.2	20.0[r] ± 0.9
Acetic acid control[†]	25.2[r] ± 5.9	11.0[r] ± 0.0	5.5[r] ± 2.0	116.0[r] ± 46.3	20.5[r] ± 3.4
1:1000	27.0[r] ± 3.2	11.0[r] ± 0.0	5.7[r] ± 1.1	134.3[r] ± 25.3	23.6[r] ± 28
1:100	27.5[r] ± 1.6	11.0[r] ± 0.0	5.7[r] ± 1.3	133.0[r] ± 20.2	23.8[r] ± 3.2

(continued)

(Table 17 continued)

Extract and concentration	Life length during study (days)	Day of first brood release	No. of broods/adult	No. of young/adult	No. of young/brood
Power plant No. 1 bottom ash					
Control	23.2[s] ± 8.2	8.6[t] ± 4.7	5.0[s] ± 2.8	84.2[s] ± 53.0	13.5[s] ± 8.5
Acetic acid control[†]	24.9[s] ± 6.9	12.0[s] ± 4.9	4.6[s] ± 2.3	72.0[s] ± 40.9	13.5[s] ± 7.5
1:1000	24.5[s] ± 4.3	12.8[s] ± 0.6	4.4[s] ± 1.5	62.1[s] ± 33.2	13.6[s] ± 5.1
1:100	21.4[s] ± 6.8	12.1[s] ± 2.1	3.7[s] ± 2.3	45.4[s] ± 42.3	10.2[s] ± 4.6
Power plant No. 1 scrubber sludge					
Control	28.0[u] ± 0.0	9.0[u] ± 0.0	6.8[u] ± 0.4	139.8[v] ± 6.2	20.7[v] ± 2.0
Acetic acid control[†]	28.0[u] ± 0.0	10.7[u] ± 2.3	6.8[u] ± 1.0	186.0[u] ± 39.2	27.0[u] ± 3.1
1:1000	27.8[u] ± 0.4	9.0[u] ± 1.1	7.2[u] ± 0.4	134.8[v] ± 25.0	18.8[v] ± 3.6
1:100	28.0[u] ± 0.0	9.0[u] ± 0.0	6.6[u] ± 0.9	151.6[v] ± 12.6	23.4[v] ± 4.0
Power plant No. 1 treated scrubber sludge					
Control	27.1[w] ± 6.0	17.22 ± 2.5	4.4[w,x] ± 1.8	102.8[w] ± 50.0	23.0[w]
Acetic acid control[†]	26.6[w] ± 6.0	18.0[w] ± 3.28	3.7[w,x] ± 1.8	64.3[w,x] ± 33.8	18.9[w]
1:1000	22.4 ± 8.7	19.5[w] ± 1.4	2.5[x] ± 2.3	43.5[w] ± 41.5	17.5[w]
1:100	25.6[w] ± 6.2	14.0[x] ± 2.4	4.7[w] ± 2.7	89.0[w,x] ± 69.5	7.8[w]
Power plant No. 2 fly ash					
Control	26.5[y] ± 4.7	11.0[y] ± 0.0	6.2[y] ± 1.6	139.4[y] ± 69.7	21.2[y] ± 8.0
Acetic acid control[†]	28.0[y] ± 0.0	11.4[y] ± 2.2	6.3[y] ± 1.4	128.1[y] ± 67.0	18.9[y] ± 8.3
1:1000	26.1[y] ± 3.8	11.6[y] ± 1.0	5.7[y] ± 1.5	109.2[y] ± 60.6	18.3[y] ± 7.5
1:100	27.4[y] ± 1.9	11.0[y] ± 0.0	6.7[y] ± 0.8	138.9[y] ± 45.5	20.5[y] ± 5.6

(continued)

(Table 17 continued)

Extract and concentration	Life length during study (days)	Day of first brood release	No. of broods/adult	No. of young/adult	No. of young/brood
Fluidized bed residue					
Control	28.0[z] ± 0.0	12.0[z] ± 0.0	6.7[z] ± 0.7	149.2[z] ± 34.7	22.2[z] ± 4.1
Hydrocloric acid content[#]	27.1[z] ± 9.0	12.0[z] ± 0.0	5.9[z] ± 1.7	169.8[z] ± 80.8	21.2[z] ± 9.6
1:1000	28.0[z] ± 0.0	12.0[z] ± 0.0	6.9[z] ± 0.57	178.0[z] ± 30.9	25.8[z] ± 4.6
1:100	27.3[z] ± 1.6	11.8[z] ± 0.6	6.1[z] ± 1.3	153.5[z] ± 77.7	23.8[z] ± 10.0
Gasification waste No. 1					
Control	26.1[aa] ± 4.8	10.0[bb] ± 0.0	6.3[aa] ± 1.7	101.8[aa] ± 32.6	15.8[aa, bb] ± 2.6
Acetic acid control[†]	27.2[aa] ± 2.5	10.7[aa] ± 1.3	6.5[aa] ± 1.5	99.9[aa] ± 21.7	15.5[bb] ± 1.6
1:1000	25.4[aa] ± 5.5	10.1[bb] ± 0.3	6.0[aa] ± 1.6	109.1[aa] ± 36.8	17.8[aa] ± 2.8
1:100	27.2[aa] ± 2.5	10.0[bb] ± 0.0	6.5[aa] ± 1.0	111.9[aa] ± 19.9	17.2[aa, bb] ± 2.0
Gasification waste No. 2					
Control	29.0[cc] ± 0.0	10.0[dd] ± 0.0	6.7[cc] ± 1.1	87.6[cc] ± 16.3	13.2[cc, dd] ± 2.8
1:1000	29.0[cc] ± 0.0	14.3[cc] ± 1.7	5.7[dd] ± 0.7	66.3[dd] ± 10.5	11.6[dd] ± 1.1
1:100	29.0[cc] ± 0.0	10.8[dd] ± 1.8	6.6[cc] ± 0.7	91.6[cc] ± 13.8	13.9[cc] ± 1.5
Gasification waste No. 3					
Control	24.9[ee] ± 6.2	12.9[ee] ± 4.7	3.6[ee] ± 1.9	36.8[ee] ± 23.2	9.7[ee] ± 3.3
Acetic acid control[†]	22.0[ee] ± 7.3	13.6[ee] ± 6.8	2.8[ee] ± 2.0	23.4[ee] ± 20.8	7.7[ee] ± 3.6
1:1000	21.1[ee] ± 6.0	12.0[ee] ± 1.8	3.1[ee] ± 1.5	26.7[ee] ± 20.0	7.9[ee] ± 3.9
1:100	21.7[ee] ± 6.8	10.6[ee] ± 1.3	3.7[ee] ± 1.5	29.5[ee] ± 16.5	7.6[ee] ± 2.2

(continued)

(Table 17 continued)

*Values given are mean ± S.E. Means with the same letter are not significantly different ($\alpha = 0.05$).

†Acetic acid, neutralized to pH 7.0 with NaOH, added at same concentration as present in the 1:100 dilution of the extract.

‡Only one brood was produced by 10 test animals.

§All young were stillborn.

#Hydrochloric acid, neutralized to pH 7.0 with NaOH, added at the same concentration as present in the 1:100 dilution of the extract.

TABLE 18. SUMMARY OF THE D. MAGNA ACUTE AND CHRONIC TOXICITY TEST RESULTS

Extract	48-h LC_{50} (% conc.)	Significance* of chronic toxicity test results at dilution	
		1:1000	1:100
Arsenic-contaminated groundwater	2.2	NS	S
Soybean process cake	No significant mortality	NS	NS
Metal processing waste	28.0	NS†	NS‡
Plater's waste	0.005	S	S
Raw shale	53.9	NS	NS
Retorted shale	70.0	NS	NS
Dye waste	0.0005	S	S
Textile waste	19.2	NS	NS
Municipal sewage sludge	7.5	S	S
Power plant No. 1 fly ash	90	NS	NS
Power plant No. 1 bottom ash	94	NS	NS
Power plant No. 1 scrubber sludge	85	NS†	NS†
Power plant No. 1 treated scrubber sludge	81.1	NS‡	NS
Power plant No. 2 fly ash	15.2	NS	NS
Fluidized bed residue	12.6	NS	NS
Gasification waste No. 1	<50% mortality	NS	NS
Gasification waste No. 2	100.0	S	NS
Gasification waste No. 3	70.0	NS	NS

*The criterion for significance was a reduction ($\alpha = 0.05$) in the mean number of young produced at either test dilution compared with the dilution water and acetic acid controls. NS, not significant; S, significant.

†Not significant when the treatment mean was compared with the dilution water control mean, but significant when compared with the acetic acid control mean.

‡Not significant when the treatment mean was compared with the acetic acid control mean, but significant when compared with the dilution water control mean.

Arsenic-Contaminated Groundwater

This material was toxic to D. magna in both acute and chronic tests. The 48-h LC_{50} concentrations were 2.2 and 1.7% (Table 16). In the chronic toxicity tests there were no significant effects on reproduction or survival at the 1:1000 dilution, but at the 1:100 dilution significant effects were found by three of the five toxicity criteria (Table 17). The mean number of young produced per adult and the mean number of young per brood were both reduced by 88% compared with the dilution water controls. Also, the mean day of first brood release significantly increased from 11.3 for the controls to 13.7 for animals in the 1:100 dilution.

Soybean Process Cake

In the first acute toxicity test, no animals were killed or immobilized after an exposure to 100% of the extract for 48 h; in the second test less than 50% were adversely affected (Table 16). Results of the chronic toxicity test also showed the low toxicity of the extract for D. magna (Table 17). There were no significant reductions in any of the toxicity values obtained for the test animals compared with the control values at either of the two test dilutions.

Metal Processing Waste

This material was toxic to D. magna in the acute toxicity tests with 48-h LC_{50}s of 28.0 and 39.0% (Table 16), but it was not toxic at the test dilutions used in the chronic toxicity tests (Table 17).

Plater's Waste

The acute toxicity test done with this material showed that it was highly toxic to D. magna. The 48-h LC_{50} was 0.005% (Table 16). Because this value is less than the 0.1% dilution used in the chronic toxicity tests, these tests were not done.

Raw Shale

This material had low toxicity to D. magna in the acute toxicity tests. The 48-h LC_{50}s were 53.9 and 64.4% (Table 16). By three of the five criteria used in the chronic toxicity tests, there were no effects of the material on the organisms (Table 17). With mean number of young per adult and mean number of young per brood as criteria, there is evidence for stimulation of reproduction at 1:100 dilution and no or little inhibition at 1:1000 dilution (Table 17).

Retorted Shale

As was the case with raw shale, retorted shale had low toxicity for D. magna in the acute toxicity tests. The 48-h LC_{50}s were 70.0 and 39.4% (Table 16). The

effects in the chronic toxicity tests were not significantly different from the control values (Table 17).

Dye Waste

This material was very toxic to D. magna. The 48-h LC_{50} was 0.0005% (Table 16). Because this value is less than the 0.1% dilution used in the chronic toxicity tests, these tests were not done.

Textile Waste

This material was moderately toxic to D. magna in the acute toxicity tests. The 48-h LC_{50}s were 19.2 and 35.5% (Table 16). In the chronic toxicity tests, there were no effects by three of the five toxicity criteria (Table 17). By the other criteria, reproduction was stimulated at the 1:100 dilution but the 1:1000 dilution had no effect.

Municipal Sewage Sludge

This material was toxic to D. magna in both the acute and chronic toxicity tests (Tables 16 and 17). The 48-h LC_{50}s were 7.5 and 3.0%.

Power Plant No. 1 Fly Ash, Bottom Ash, and Scrubber Sludge

These materials had low toxicity to D. magna in the acute toxicity tests (Table 16). The 48-h LC_{50}s for the fly ash, bottom ash, and scrubber sludge were 90 and 69%, 94 and 60%, and 85 and 60%, respectively. They also had no effect in the chronic toxicity tests (Table 17).

Power Plant No. 1 Treated Scrubber Sludge

This material had low toxicity to D. magna in the acute toxicity tests. The 48-h LC_{50}s were 81.1 and 95.6% (Table 16). In the chronic toxicity tests, there was no effect by either the 1:100 or 1:1000 dilution on D. magna reproduction (Table 17) when compared with the acetic acid control. However, a difference was noted between the dilution water control and the 1:1000 dilution.

Power Plant No. 2 Fly Ash

In the acute toxicity tests, this material showed a moderate toxicity to D. magna. The 48-h LC_{50}s were 15.2 and 11.6% (Table 16). This material was not toxic at either test dilution in the chronic assay, however (Table 17).

Fluidized Bed Residue

This material was moderately toxic to D. magna in the acute toxicity tests. The 48-h LC_{50}s were 12.6 and 25.0% (Table 16). The material was not toxic at either test dilution in the chronic toxicity tests (Table 17).

Gasification Wastes No. 1–3

These materials had low toxicities to D. magna in the acute toxicity tests (Table 17). Toxic effects occurred only with full or near full strength solutions. Results of the chronic toxicity tests (Table 17) showed no effects of these wastes except for the 1:1000 dilution of waste No. 2 (Table 17). But since there were no significant effects with this material at 1:100 dilution, the apparent effects with the higher dilution become less important in evaluating the toxicity of waste No. 2 than if significant effects were found for both dilutions.

DISCUSSION

In these chronic toxicity tests, the effects of the test materials on D. magna were determined by the use of five population characteristics as toxicity criteria. Of these criteria, the most consistently sensitive were the number of young per brood and the number of young per adult; survival time, the day of first brood release, and the number of broods produced were affected less by the extracts. Since the total number of young produced per adult is in part a function of the number of young per brood, the former was chosen for convenience to assess toxicity. Results from these studies and from other studies with the 28-day chronic toxicity test with D. magna support the following conclusions:

(1) The mean number of young produced per brood and the mean total number of young produced by each female are the most consistently sensitive population characteristics of those tested to chemical toxicity.

(2) With these two characteristics as toxicity criteria, the test can be used reliably to determine the maximum concentration of a chemical that produces no significant observed toxic effects and the minimum concentration that produces such effects. To do so, however, requires more than the two test concentrations used in the present screening tests.

The criterion selected to identify extracts as toxic to D. magna and as potentially hazardous to aquatic organisms was a significant reduction ($\alpha = 0.05$) in the mean number of young produced by each D. magna during the 28-day test period compared with the number produced by organisms exposed to the dilution water and acid controls. By this criterion, 4 of the 18 tested extracts, namely, arsenic-contaminated groundwater, plater's waste, dye waste, and municipal sewage sludge, would be classified as potentially

hazardous (Table 18). Of the remaining extracts, only the metal processing waste and power plant No. 1 untreated and treated scrubber sludge showed any significant effects, but only when the results with the two test dilutions were compared with either the acetic acid controls or dilution water controls (Table 18).

On the basis of screening test results, it is not possible to definitely determine if waste would be hazardous to a natural population or community of aquatic organisms. There are limitations with the extrapolation of laboratory results based on single species tests to the prediction of effects on natural ecosystems. The single-species test systems are simple compared with complex natural ecosystems. Thus, even though a significant toxic effect can be demonstrated in the laboratory, under natural conditions it may be mitigated, modified, or even magnified by the chemical, physical, and biological interactions of the ecosystem. However, D. magna is sensitive to many classes of toxic chemicals, and thus results of toxicity tests with this species can be regarded as conservative predictors of potential hazard to other aquatic species.

Because of limitations with the EP used for the D. magna chronic toxicity tests, some toxic wastes may not have been identified. For example, all volatile chemicals were probably lost during the extraction process. Also, the renewal-type test procedure used in the chronic toxicity tests is not as efficient as continuous flow-through test systems for detecting nonpersistent chemicals.

In summary, the chronic test results can be used to identify only those wastes that are potentially hazardous. More in-depth tests under site-specific conditions with several test organisms would be necessary to determine conclusively if a waste was hazardous to a particular aquatic ecosystem.

Comparison of results from the second set of acute toxicity tests, which were done 28 days after the first ones, with results from the first set determined that the toxicities of some extracts increased during storage, whereas those of others decreased (Table 16), but the differences are probably not statistically significant for arsenic-contaminated groundwater, soybean process cake, and metal processing waste. Data for the remaining extracts are insufficient for statistical analysis.

RECOMMENDATIONS

The 28-day chronic toxicity test with D. magna can detect toxic materials in extracts (Table 18). However, reproduction of D. magna is affected by food supply. Some neutralized test extracts contained high concentrations of acetate, which can stimulate bacterial reproduction. Bacteria are food for D. magna. Acetate can serve as food for bacteria, and this increased bacterial level could indirectly stimulate D. magna reproduction. Of the 15 tests in which both an acetate and dilution water control were used, 12 showed no significant differences in reproduction and 3 showed slightly higher fecundity in the acetate controls relative to dilution water controls. These results may be explained by the concentration of acetate in the water. Results of

some of our other research suggest that low concentrations of acetate stimulate reproduction, but that concentrations ≥ 0.1% are toxic. Nevertheless, interactions of acetate with other toxic chemicals in the waste materials are not known. These interactions could have synergistic or antagonistic toxic effects on aquatic organisms, which could make the interpretation of the test results more difficult. Therefore, additional work should be conducted to determine the effects acetic acid has on aquatic organisms, and, if necessary, an EP to determine aquatic toxicity should be developed to correct this problem.

REPRODUCIBILITY OF CHRONIC TOXICITY TEST

There is little information on the reproducibility of results obtained with the 28-day D. magna chronic toxicity test, which was the test used in the above screening experiments. Therefore, we did a study to determine if this chronic toxicity test will yield reproducible results.

We chose the azaarene, acridine, as the test material because of our experience with it in testing coal conversion products and wastes. The materials and methods were those used in the above screening tests (Appendix D), except that a series of test dilutions were used instead of just two, and 20 animals were used for each test concentration instead of 10. In this study, "no observed effects" concentrations (NOECs) were determined, which are defined as the highest concentration of a chemical that causes no statistically significant observed effects on the test organisms compared with the controls.

The NOECs ranged between 0.4 and 1.6 mg/l of acridine in the four tests (Table 19). The NOECs for survival, age at onset of reproduction, and number of broods per

TABLE 19. NOECs FROM FOUR TESTS ON THE CHRONIC TOXICITY OF ACRIDINE TO D. MAGNA

Toxicity criterion	NOEC (mg/l) for test No.			
	1	2	3	4
Survival	0.8	1.6	0.4	0.8
Occurrence of primiparous instar	0.8	0.8	0.8	0.8
Age at onset of reproduction	0.8	0.8	0.4	0.4
No. of broods/female	0.8	0.4	0.4	0.8
No. of young/brood	0.4	0.4	0.4	0.4
No. of young/female	0.4	0.4	0.4	0.4

female varied significantly among the four tests, but the NOECs for occurrence of primiparous instar (when young are first present in the female's brood chamber), number of young per brood, and number of young per female did not vary at all in the four tests. The latter two criteria were the most sensitive for estimation of the NOEC, since their NOEC (0.4 mg/l) was less than the value for occurrence of the primiparous instar (0.8 mg/l).

The responses of D. magna to various acridine concentrations were usually not the same among the four tests (Table 20). At 3.2 mg/l the results were identical among the tests for five of the six toxicity criteria, but at this concentration all means were zero since reproduction was completely inhibited by acridine. Results of the study by Canton and Adema[12] on the reproducibility of a 21-day D. magna chronic toxicity test appear to support our findings. Variations in the inhibition of reproduction were 20% or greater in their duplicate tests with six compounds; however, NOECs appeared to be reproducible in most of their experiments. Our interpretation of their results is tentative since statistical analyses were not provided.

In conclusion, the D. magna static-renewal chronic toxicity test using either the number of young per brood or number of young per female as the toxicity criterion is a reproducible and sensitive test.

TABLE 20. VARIABILITY IN RESPONSE OF D. MAGNA TO ACRIDINE IN FOUR CHRONIC, STATIC-RENEWAL TESTS*

Toxicity criterion	Significance of variation at acridine concentration (mg/l)						
	0.0 (control)	0.0 (methanol control)	0.2	0.4	0.8	1.6	3.2
Survival	NS	NS	NS	S	S	S	S
Occurrence of primiparous instar	S	S	S	S	S	S	NS
Age at onset of reproduction	S	S	S	S	S	S	NS
No. of broods/female	S	S	S	S	S	S	NS
No. of young/brood	S	S	S	S	S	S	NS
No. of young/female	S	S	S	S	S	S	NS

*NS, not significant at $\alpha = 0.05$; S, significant.

SECTION 7

PHYTOTOXICITY

PROBLEM DEFINITION

The problem posed to us by EPA was to develop and evaluate short-term screening bioassay tests for determining whether materials extracted from a waste, as determined by the EP, would be harmful to terrestrial plants if the leachate was used as irrigation water. Toward this end short-term root elongation and seedling growth assays were done on 17 extracts and one groundwater sample. Problems were anticipated because of reports in the literature that acetic acid/acetate ion, even at relatively low levels, can be phytotoxic.[13]

METHODOLOGY

Short-term (48- and 72-h) root (radicle) elongation tests were performed with radish and sorghum in a controlled environment. Longer-term (2- and 3-week) seedling growth studies were carried out in pots under greenhouse conditions with wheat and soybean. Parameters measured were root length in the short-term tests and root and shoot dry weight (or length in some cases) in the longer-term tests. Treated plants were compared with plants grown in distilled water in the case of the short-term tests or a plant nutrient solution in the longer-term study. Treated plants received a 10% concentration of the EP extract (diluted with nutrient solution) in most of the long-term tests to simulate a tenfold dilution that EPA assumes will occur as water drains from a natural landfill to groundwater. This groundwater could then be used to irrigate crops. However, following seedling growth studies with acetic acid, we decided to dilute the fluidized bed residue and the power plant No. 1 treated scrubber sludge samples to 2 and 8%, respectively, to reduce phytotoxic effects of acetic acid. In the short-term tests, treated plants received a series of concentrations of the EP extracts. Preliminary results from radish and sorghum treated with different concentrations of acetic acid showed inhibited plant growth only when the acetic acid concentrations exceeded ≃5 ml of 0.5 N acetic acid per l (2.5 meq/l). The highest concentration to be tested was chosen by diluting the volume of acetic acid added during extraction to approximately the 5 ml/l toxicity threshold.

The plant species selected for these tests are important agriculturally and represent the two major classes (monocotyledons and dicotyledons) of flowering plants. Because of the difference in growth habits of these two classes of plants, they may respond differently

to various chemicals in their environment. Of course, this is also true to a lesser extent between individual species of the same class, but it was not feasible to test large numbers of many different species. More detailed methods are given in Appendices E and F.

RESULTS AND DISCUSSION

Arsenic-contaminated groundwater was highly phytotoxic, showing 33% growth reduction of radish roots (radicles) even at a 2% concentration. Higher concentrations (10 and 25%) reduced growth 70 and 59%, respectively. However, at the highest dilution (0.1%) there was a slight stimulation of root growth.

Data from other tests in which extracts were obtained by the EPA EP are presented in Tables 21 and 22. Table 23 presents data on acetic acid effects (see "Problem Areas"). Table 24 is a summary of the results presented in these three tables.

Of the coal processing wastes, three (power plant No. 2 fly ash, gasification waste No. 1, and fluidized bed residue) were not toxic. Retorted shale and power plant No. 1 treated scrubber sludge reduced growth of the monocotyledon species (sorghum and wheat) in both types of tests. The latter inhibited both root and shoot growth of wheat; the former inhibited only root growth. Raw shale caused a growth reduction of 14% in sorghum roots. These three extracts were phytotoxic at less than a tenfold dilution.

Power plant No. 1 scrubber sludge was toxic to radish seeds in the root elongation test even at a 10% concentration, but the same concentration was not toxic to sorghum. Power plant No. 1 bottom ash and fly ash were only slightly toxic to either radish or sorghum at concentrations exceeding 10%. In the seedling growth studies power plant No. 1 fly ash caused a slight but significant reduction of wheat root weight but not shoot weight.

Results of the other two gasification wastes showed the waste designated No. 2 to be the only one of the three which was toxic at a concentration less than 10%. This effect was seen in the seedling growth study with soybean roots. Gasification waste No. 3 caused 22 and 12% growth reductions of radish and sorghum roots, respectively, at 100% concentration.

Municipal sewage sludge was the only one of six industrial wastes that was not toxic. Soybean process cake significantly inhibited root growth of wheat and sorghum. Although the latter monocotyledon species showed no effect at 50% concentration after 72 h, the waste was toxic to wheat exposed for 2 weeks even though the solution was more dilute (10% concentration). Metal processing waste also affected monocotyledon root growth significantly.

TABLE 21. RADISH AND SORGHUM RADICLE LENGTHS*

Extract	Concentration (%)	Seed type	Root length† (mm)	% Growth reduction
Arsenic-contaminated groundwater	10	radish	8 ± 6	70‡
	5		11 ± 7	59‡
	2		18 ± 8	33‡
	0.1		31 ± 12	0
	control		27 ± 14	—
Soybean process cake	75	radish	23 ± 10	0
	control		22 ± 10	—
	75	sorghum	23 ± 10	15‡
	50		28 ± 14	0
	control		27 ± 12	—
Metal processing waste	4	radish	27 ± 13	4
	control		28 ± 13	—
	4	sorghum	29 ± 13	12‡
	control		33 ± 13	—
Plater's waste	5	radish	14 ± 6	50‡
	2		22 ± 8	21‡
	control		28 ± 13	—
	0.5		33 ± 12	0
	control		31 ± 14	—
	5	sorghum	12 ± 5	0
	control		11 ± 5	—
Raw shale	4	radish	23 ± 12	4
	control		24 ± 12	—
	4	sorghum	25 ± 11	14‡
	2		29 ± 12	0
	control		29 ± 12	—
Retorted shale	2	radish	25 ± 11	0
	control		25 ± 13	—
	2	sorghum	25 ± 11	14‡
	1		25 ± 11	14‡
	control		29 ± 12	—
	0.5		42 ± 18	0
	control		38 ± 18	—

(continued)

(Table 21 continued)

Extract	Concentration (%)	Seed type	Root length† (mm)	% Growth reduction
Dye waste	10	radish	21 ± 11	16‡
	5		23 ± 10	8
	control		25 ± 13	—
	10	sorghum	9 ± 5	18‡
	5		9 ± 4	18‡
	control		11 ± 5	—
	0.5		41 ± 17	0
	control		38 ± 16	—
Textile waste	5	radish	22 ± 9	21‡
	2		27 ± 11	4
	control		28 ± 13	—
	5	sorghum	30 ± 11	9‡
	control		33 ± 13	—
Municipal sewage sludge	20	radish	35 ± 12	0
	control		33 ± 12	—
	20	sorghum	28 ± 9	3
	control		29 ± 12	—
Power plant No. 1 fly ash	30	radish	27 ± 15	13‡
	control		31 ± 16	—
	30	sorghum	15 ± 13	12
	control		17 ± 15	—
Power plant No. 1 bottom ash	100	radish	21 ± 11	5
	control		22 ± 12	—
	100	sorghum	20 ± 12	13‡
	control		23 ± 13	—
Power plant No. 1 scrubber sludge	10	radish	16 ± 11	20‡
	control		20 ± 15	—
	10	sorghum	38 ± 30	3
	control		39 ± 28	—

(continued)

(Table 21 continued)

Extract	Concentration (%)	Seed type	Root length† (mm)	% Growth reduction
Power plant No. 1 treated scrubber sludge	8	radish	32 ± 10	6
	control		34 ± 12	—
	8	sorghum	30 ± 10	9‡
	4		33 ± 12	0
	control		33 ± 13	—
Power plant No. 2 fly ash	100	radish	32 ± 10	6
	control		34 ± 12	—
	100	sorghum	31 ± 11	3
	control		32 ± 11	—
Fluidized bed residue	2	radish	34 ± 13	6
	control		36 ± 15	—
	2	sorghum	40 ± 14	4
	control		42 ± 16	—
Gasification waste No. 1	100	radish	21 ± 10	0
	control		21 ± 12	—
	100	sorghum	21 ± 11	9
	control		23 ± 16	—
Gasification waste No. 2	100	radish	27 ± 12	0
	control		25 ± 12	—
	100	sorghum	24 ± 13	0
	control		19 ± 13	—
Gasification waste No. 3	100	radish	24 ± 12	22‡
	50		32 ± 15	0
	control		31 ± 17	—
	100	sorghum	22 ± 11	12‡
	control		25 ± 9	—

*Radish and sorghum were grown at 25°C in the dark for 48 and 72 h, respectively. Controls were grown with distilled water and compared with plants treated with the indicated concentration of extract.

†Values given are mean ± S.D.

‡Significantly different from controls at a 5% probability level.

TABLE 22. WHEAT AND SOYBEAN SHOOT AND ROOT DRY WEIGHTS OR LENGTHS*

Extract	Concentration (%)	Seed type	Root		Shoot	
			Weight† (g)	% Growth reduction	Weight† (g)	% Growth reduction
Power plant No. 1 fly ash	10	wheat	0.28 ± 0.04	15‡	0.83 ± 0.10	5
	control		0.33 ± 0.05	—	0.87 ± 0.09	—
Power plant No. 1 scrubber sludge	10	wheat	0.08 ± 0.01	0	0.12 ± 0.01	8
	control		0.08 ± 0.01	—	0.13 ± 0.02	—
Power plant No. 1 fly ash	10	soybean	0.05 ± 0.02	17	0.42 ± 0.02	2
Power plant No. 1 scrubber sludge	10	soybean	0.09 ± 0.03	0	0.41 ± 0.06	5
	control	soybean	0.06 ± 0.03	—	0.43 ± 0.07	—
Gasification waste No. 1	10	wheat	0.08 ± 0.01	0	0.19 ± 0.03	0
Soybean process cake	10	wheat	0.07 ± 0.01	13‡	0.21 ± 0.03	0
	control	wheat	0.08 ± 0.01	—	0.18 ± 0.03	—
Gasification waste No. 1	10	soybean	0.19 ± 0.03	0	0.31 ± 0.05	0
Soybean process cake	10	soybean	0.18 ± 0.05	0	0.29 ± 0.04	0
	control	soybean	0.18 ± 0.04	—	0.29 ± 0.04	—

(Continued)

(Table 22 continued)

Extract	Concentration (%)	Seed type	Root		Shoot	
			Weight† (g)	% Growth reduction	Weight† (g)	% Growth reduction
Gasification waste No. 2	10	wheat	0.09 ± 0.01	0	0.28 ± 0.03	3
Power plant No. 1 bottom ash	10	wheat	0.09 ± 0.01	0	0.31 ± 0.02	0
	control	wheat	0.09 ± 0.02	—	0.29 ± 0.04	—
Gasification waste No. 2	10	soybean	0.08 ± 0.01	20‡	0.17 ± 0.03	0
Power plant No. 1 bottom ash	10	soybean	0.09 ± 0.02	10	0.18 ± 0.04	0
	control	soybean	0.10 ± 0.02	—	0.17 ± 0.03	—
Gasification waste No. 3	10	wheat	0.0518 ± 0.0046	3	0.1719 ± 0.0139	0
Plater's waste§	10	wheat	0.0478 ± 0.0065	11‡	0.1200 ± 0.0149	29‡
	control	wheat	0.0535 ± 0.0043	—	0.1700 ± 0.0089	—
Gasification waste No. 3	10	soybean	0.0704 ± 0.0149	1	0.1818 ± 0.0189	7
Plater's waste§	10	soybean	0.0163 ± 0.0045	77‡	0.0716 ± 0.0211	63‡
	control	soybean	0.0712 ± 0.0209	—	0.1945 ± 0.0255	—

(Continued)

(Table 22 continued)

Extract	Concentration (%)	Seed type	Root		Shoot	
			Weight† (g)	% Growth reduction	Weight† (g)	% Growth reduction
Metal processing waste§	10	wheat	0.0795 ± 0.013	17‡	0.1668 ± 0.0253	7
Raw shale§	10	wheat	0.1193 ± 0.0276	0	0.1810 ± 0.0309	0
	control	wheat	0.0963 ± 0.0194	—	0.1785 ± 0.0257	—
Metal processing waste§	10	soybean	0.0862 ± 0.0229	0	0.1392 ± 0.0239	0
Raw shale§	10	soybean	0.0774 ± 0.0096	9	0.1217 ± 0.0110	7
	control	soybean	0.0851 ± 0.0136	—	0.1315 ± 0.0165	—
Retorted shale§	10	wheat	0.0632 ± 0.0094	16‡	0.1340 ± 0.0206	3
Dye waste	10	wheat	0.0423 ± 0.0059	44‡	0.0806 ± 0.0130	42‡
	control	wheat	0.0750 ± 0.0063	—	0.1382 ± 0.0193	—
Retorted shale§	10	soybean	0.0831 ± 0.0144	4	0.1538 ± 0.0201	0
Dye waste	10	soybean	0.0298 ± 0.0083	66‡	0.0930 ± 0.0360	26‡
	control	soybean	0.0868 ± 0.0234	—	0.1252 ± 0.0190	—

(Continued)

(Table 22 continued)

Extract	Concentration (%)	Seed type	Root		Shoot	
			Length†,# (cm)	% Growth reduction	Length†,# (cm)	% Growth reduction
Fluidized bed residue	2	wheat	9 ± 4	0	16 ± 7	0
Municipal sewage sludge	10	wheat	10 ± 3	0	18 ± 7	0
	control	wheat	8 ± 3	—	15 ± 6	—
Fluidized bed residue	2	soybean	6 ± 2	0	9 ± 4	0
Municipal sewage sludge	10	soybean	6 ± 3	0	7 ± 4	0
	control	soybean	5 ± 3	—	7 ± 3	—
Power plant No. 2 fly ash	10	wheat	14 ± 2	0	22 ± 3	0
Power plant No. 1 treated scrubber sludge	8	wheat	12 ± 1	14‡	18 ± 3	10‡
	control	wheat	14 ± 2	—	20 ± 3	—
Power plant No. 2 fly ash	10	soybean	10 ± 3	0	12 ± 2	0
Power plant No. 1 treated scrubber sludge	8	soybean	9 ± 2	0	12 ± 2	0
	control	soybean	8 ± 2	—	11 ± 2	—

(Continued)

(Table 22 continued)

*Plants were grown in sand culture for 2 weeks under greenhouse conditions and watered with a natural solution (controls) or with the indicated extract diluted with the nutrient solution. Concentration indicates the percent extract in the solution added to the plants. When two extracts were run concurrently, control results are not shown with each but are placed after the second extract.

†Values given are mean ± S.D.

‡Significantly different from controls at a 5% probability level.

§Possible acetic acid toxicity interference at a 10% concentration.

#After a comparison of weight versus length for determination of effects, measurement of length was found to be faster and more consistent than weight. Therefore, for the rest of the extracts tested, length was the parameter observed.

Textile waste and plater's waste affected growth of the dicotyledon species (radish and soybean), and plater's waste also inhibited wheat root and shoot growth. Only one of the extracts (dye waste) showed toxic effects in all the tests. This could have been due to high concentrations of heavy metals, especially Cu, as indicated by analytical results.

Only one extract which caused growth inhibition (gasification waste No. 3) was not toxic in any test at a tenfold dilution. All of the others inhibited growth in at least one of the tests at that dilution or below it. Four (plater's waste, metal processing waste, raw shale, and retorted shale) had possible acetic acid interference at tenfold dilution. Fluidized bed residue was not tested at a tenfold dilution because of the acetic acid problem.

PROBLEM AREAS

We are quite confident in the root elongation test results. Radish and sorghum seeds are best suited for use in the original design of the germination chamber, but the basic chamber can be custom-built to accommodate different seed types. The main drawback in the results was caused by toxic interference of acetic acid. Table 23 summarizes the effect of acetic acid on radish and sorghum seeds in the root elongation bioassay and wheat and soybean in the seedling growth study. Metal waste, plater's waste, raw shale, retorted shale, textile waste, fluidized bed residue, and power plant No. 1 treated scrubber sludge could not be tested at even the 10% concentration because of acetic acid interference. As this problem is not inherent to the bioassay procedure, we maintain that the technique is acceptable for initial screening. When acetic acid concentrations are too high, it may be necessary to: (1) use more dilute concentrations and consider tests valid only if there is growth inhibition; (2) find suitable plant species which are less sensitive to acetic acid at concentrations required by the EP; or (3) use another leaching method.

As is normally expected with biological systems, variability of the measured parameters was high within and between tests. We were able to reduce variability from $\simeq$75% in earlier tests to $\simeq$40% in later tests by sieving seeds to obtain uniform size, stabilizing temperature at 25°C, and staying as close to the designated incubation time as possible. Controls should be run during each test, and care must be taken to treat controls and treatment plants exactly the same during each test to minimize experimental error.

Sand was chosen for the seedling growth study because various types of soil influence toxicity of phytotoxic substances depending on the amount of organic matter present.[14] However, the effect of soil organic matter on phytotoxic effects of solid waste extracts or other potentially toxic substances is an area that needs further research. This is an important consideration in assessing the hazards of toxic substances to the terrestrial environment and in verifying these types of screening bioassays.

TABLE 23. EFFECT OF ACETIC ACID ON WHEAT AND SOYBEAN PLANTS IN THE LONG-TERM STUDY AND ON RADISH AND SORGHUM SEEDS IN THE ROOT ELONGATION BIOASSAY

Seed type	Acetic acid* (meq/l)	Root		Shoot	
		Length† (cm)	% Growth reduction	Length† (cm)	% Growth reduction
Wheat	10	5 ± 2	58‡	14 ± 5	36‡
	5	9 ± 2	25‡	19 ± 5	14‡
	2.5	8 ± 3	33‡	17 ± 6	23‡
	0.5	13 ± 4	0	19 ± 6	14‡
	control	12 ± 4	—	22 ± 8	—
Soybean	10	5 ± 1	38‡	6 ± 2	40‡
	5	6 ± 2	25‡	9 ± 2	10‡
	2.5	6 ± 2	25‡	8 ± 3	20‡
	0.5	7 ± 2	13‡	11 ± 2	0
	control	8 ± 2	—	10 ± 3	—
		Length† (mm)			
Radish	10	23 ± 9	43‡		
	5	34 ± 13	15‡		
	2.5	44 ± 16	0		
	control	40 ± 16	—		
Sorghum	10	24 ± 8	29‡		
	5	31 ± 9	9‡		
	2.5	31 ± 12	9		
	control	34 ± 14	—		

*pH adjusted to pH 5.0 with 1 N NaOH.

†Values given are mean ± S.D.

‡Significantly different from controls at a 5% probability level.

TABLE 24. SUMMARY OF RESULTS FROM THE ROOT ELONGATION BIOASSAY AND THE SEEDLING GROWTH STUDIES*

Extract	Seed type	Concentration (%)	Toxic effects		Acetic acid (meq/l)
			Root	Shoot	
Acetic acid	radish	2	yes	—	
		1	yes	—	
		0.5	no	—	
	sorghum	2	yes	—	
		1	yes	—	
		0.5	no	—	
	wheat	2	yes	yes	
		1	yes	yes	
		0.5	yes	yes	
		0.1	no	yes	
	soybean	2	yes	yes	
		1	yes	yes	
		0.5	yes	yes	
		0.1	yes	no	
Soybean process cake	radish	75	no	—	2.80
	sorghum	75	yes	—	2.80
		50	no	—	1.92
	wheat	10	yes	no	0.38
	soybean	10	no	no	0.38
Metal processing waste†	radish	4	no	—	2.82
	sorghum	4	yes	—	2.82
	wheat	10	yes	no	7.05
	soybean	10	no	no	7.05
Plater's waste†	radish	5	yes	—	4.67
		2	yes	—	1.87
		0.5	no	—	0.47
	sorghum	5	no	—	4.67
	wheat	10	yes	yes	9.35
	soybean	10	yes	yes	9.35
Raw shale†	radish	4	no	—	2.74
	sorghum	4	yes	—	2.74
		2	no	—	1.37
	wheat	10	no	no	6.85
	soybean	10	no	no	6.85

(Continued)

(Table 24 continued)

Extract	Seed type	Concentration (%)	Toxic effects		Acetic acid (meq/l)
			Root	Shoot	
Retorted shale†	radish	2	no	—	2.0
	sorghum	2	yes	—	2.0
		1	yes	—	1.0
		0.5	no	—	0.5
	wheat	10	yes	no	10
	soybean	10	no	no	10
Dye waste†	radish	10	yes	—	2.65
		5	no	—	1.32
	sorghum	10	yes	—	2.65
		5	yes	—	1.32
		0.5	no	—	0.13
	wheat	10	yes	yes	2.65
	soybean	10	yes	yes	2.65
Textile waste	radish	5	yes	—	5.0
		2	no	—	2.0
	sorghum	5	yes	—	5.0
Municipal sewage sludge	radish	20	no	—	2.50
	sorghum	20	no	—	2.50
	wheat	10	no	no	1.25
	soybean	10	no	no	1.25
Power plant No. 1 fly ash	radish‡	30	yes	—	2.85
		10	no	—	0.95
	sorghum‡	30	no	—	2.85
	wheat§	10	yes	no	0.95
	soybean§	10	no	no	0.95
Power plant No. 1 bottom ash	radish	100	no	—	1.30
	sorghum#	100	yes	—	1.30
	wheat	10	no	no	0.13
	soybean	10	no	no	0.13
Power plant No. 1 scrubber sludge	radish	10	yes	—	2.4
	sorghum	10	no	—	2.4
	wheat	10	no	no	2.4
	soybean	10	no	no	2.4

(Continued)

(Table 24 continued)

Extract	Seed type	Concentration (%)	Toxic effects		Acetic acid (meq/l)
			Root	Shoot	
Power plant No. 1 treated scrubber sludge†	radish	8	no	—	2.80
	sorghum	8	yes	—	2.80
		4	no	—	1.40
	wheat	8	yes	yes	2.80
	soybean	8	no	no	2.80
Power plant No. 2 fly ash	radish	100	no	—	2.50
	sorghum	100	no	—	2.50
	wheat	10	no	no	0.25
	soybean	10	no	no	0.25
Fluidized bed residue†	radish	2	no	—	2.0
	sorghum	2	no	—	2.0
	wheat	2	no	no	2.0
	soybean	2	no	no	2.0
Gasification waste No. 1	radish	100	no	—	0
	sorghum	100	no	—	
	wheat	10	no	no	
	soybean	10	no	no	
Gasification waste No. 2	radish	100	no	—	0
	sorghum	100	no	—	
	wheat	10	no	no	
	soybean	10	yes	no	
Gasification waste No. 3	radish	100	yes	—	2.7
		50	no	—	1.35
	sorghum	100	yes	—	2.7
	wheat	10	no	no	0.27
	soybean	10	no	no	0.27

*Significant difference was calculated with standard t-test comparing results of treated plants with results of control (Tables 21 and 22).

†Possible acetic acid interference at a 10% concentration in long-term studies.

‡Plants used in root elongation bioassay.

§Plants used in seedling growth studies.

#Since significant growth reduction was marginal at the 100% concentrations, further tests with more dilute solutions were not performed.

While these data are difficult to interpret in terms of what extracts should be considered nonhazardous to the environment, they do illustrate the complexity of developing a screening protocol for potentially phytotoxic substances. Because of the variability among plant species in the way they respond to their environment, large numbers of species should be tested. However, even with tests employing a limited number of species and conditions, such as in the test presented here, potentially hazardous chemicals may be identified or flagged for further testing. We feel that materials which proved to be significantly different from controls in at least one of the tests are, in fact, potentially hazardous. A final determination cannot be made from terrestrial studies alone, however, but rather from an overview of the results of all tested areas — mutagenic, aquatic, and analytical.

METHODOLOGY DEVELOPMENT

Radicle Elongation Chamber Design

It became evident after initial radicle elongation tests in which the seeds were germinated in petri dishes that a more efficient and reliable method would have to be developed. In petri dishes seedlings grew in a coiled pattern, so that measuring them was difficult and time-consuming. Statistical analysis of radicle elongation data indicated that a minimum of 150 seedlings would be needed to demonstrate subtle treatment effects. The time required to measure 150 seedlings was about 5 h, and accuracy was poor. A radicle elongation chamber was developed which permitted more accurate measurements in about one-tenth the time required when petri dishes were used. This chamber, described in detail in Appendix E, was designed to stand vertically with seeds and moistened blotter paper sandwiched between two sheets of Plexiglas (Figure E-1). This resulted in downward, straight growth of the radicles, enabling faster, more precise measurement than was possible previously. This method was used successfully throughout the screening tests and was perhaps the most important and useful technique developed during the phytotoxicity studies.

Seedling Growth Studies

Like the radicle elongation tests, a method for the longer-term seedling growth studies was required that could be easily duplicated at other research facilities. Therefore, we chose to use sand as a growth medium and a very simple water and extract application technique. Details of the methods are described in Appendix F. Soil and other artificial organic media were not used partly because of anticipated standardization problems and partly because organic matter contained in soil has been shown to have an attenuating effect on certain phytotoxic chemicals, which would have confounded the interpretation of results. The sand culture medium proved very successful with the wheat but only moderately successful with soybean. Soybean plants grown in sand culture did not appear as healthy as we would have preferred. A brownish discoloration of the roots led us to believe that there was some evidence of toxicity even in the control plants.

Because of the large number of extracts needing routine screening tests and other problems caused by acetic acid, we did not try to improve the method for growing soybeans until very recently. However, recent tests in which soybeans were grown hydroponically have been very successful. We suggest that this method should be considered for routine screening. Extracts to be tested can be added to the nutrient media and also sprayed on the leaves as a mist. In our test, bubbling air slowly through the nutrient medium was necessary for normal root development.

In our sand cultures one of the problems was how to apply uniform amounts of extract to the plants in a simple and inexpensive way. Originally we used a compressed air system, plastic tubing, plastic bottles, and Teflon-coated valves to apply the extracts to a number of pots simultaneously. After each test, tubing and bottles had to be replaced and valves cleaned to prevent contamination of subsequent extracts. Eventually this method was discarded and extracts were measured in a graduated glass beaker and poured directly onto the sand surface. A squeeze-type atomizer was used for foliar application. This method proved to be adequate, with less time and expense required for cleanup and replacement of tubing and bottles.

Effects of Temperature on the Growth of Radish and Sorghum Seedlings

These studies were performed because of diff rences observed between growth of control plants during separate experiments. Since we ran a control with each test, this was no real problem as far as interpretation of test results. However, there was a need to establish that differences between controls were due primarily to controllable variables such as temperature and to a lesser extent to individual variability within plant species. We had reason to believe that the temperature of our growth chamber had been affected during some of our tests because of the installation of an exhaust fan in the growth chamber. We were also concerned that the temperatures at different levels in the growth chamber could be different due to operation of the exhaust fan. Subsequent tests with continuously recording multipoint thermocouples, however, showed very even distribution of temperature throughout the chamber.

Four separate tests with radish and sorghum were run at 25°C. In these tests care was taken to maintain a constant temperature in all tests, and all radish seeds were allowed to grow exactly 48 h and all sorghum exactly 72 h (Table 25). In this way the innate variability in population was determined. Population means for sorghum ranged from 30 to 34 mm (11.8% difference), and population means for radish ranged from 33 to 34 (only a 3% difference). Thus innate differences within populations could indeed account for some of the variability between test runs. Other possible causes of variability were discussed earlier. These data generally support results from single test runs in which differences between treatments and controls of about 12% or less were usually not statistically significant.

In another series of tests with radish and sorghum, temperature was varied at 5° increments between 15 and 35°C (Figure 11). Both radish and sorghum grew least at 15°C (6 mm and 0 mm, respectively), with increasing growth rates up to a temperature of 30°C

TABLE 25. VARIABILITY IN POPULATIONS OF RADISH AND SORGHUM SEEDS*

Seed type	Root length (mm)
Radish	33 ± 12
	34 ± 12
	34 ± 12
	34 ± 12
Sorghum	34 ± 14
	32 ± 11
	33 ± 13
	30 ± 13

*Multiple tests were run at 25°C but at different times. Root length was measured after incubation in a controlled environmental chamber in the dark. Values given are mean ± S.D.

(40 mm for radish and 46 mm for sorghum). Increasing the temperature from 30 to 35°C reduced growth in radish by 55% but had no effect on sorghum. Based on these data the best temperature for radish and sorghum growth is approximately 25°C, and we recommend that further routine bioassay tests with radish and sorghum be conducted at 25°C.

Validation Tests with Known Phytotoxic Substances

Seven known herbicides were tested for phytotoxicity by use of our routine root elongation tests with radish (Table 26). Concentrations of the various herbicides used were "maximum permissible TEP elutriate concentrations" listed in the March 24, 1978, draft of section 3001 of EPA's Criteria, Identification, Methods, and Listings of Hazardous Wastes. All but one of the herbicides significantly inhibited root growth at the concentrations used. Picloram was borderline, with an 11% growth inhibition (not significantly different from controls); it is known to initially affect mostly stem growth, with only an indirect effect on root growth as the plant matures. We were surprised that diquat reduced growth, since it is generally effective only in the light. However, both diquat and dalapon were borderline in their effects, each with only a 12% growth reduction. We can say conclusively that silvex, Fenac, dichlobenil, and 2,4,-D were phytotoxic to radish. The need for testing with more than one species of plant and for longer time periods is apparent here. Time did not permit further tests with our other test species, however.

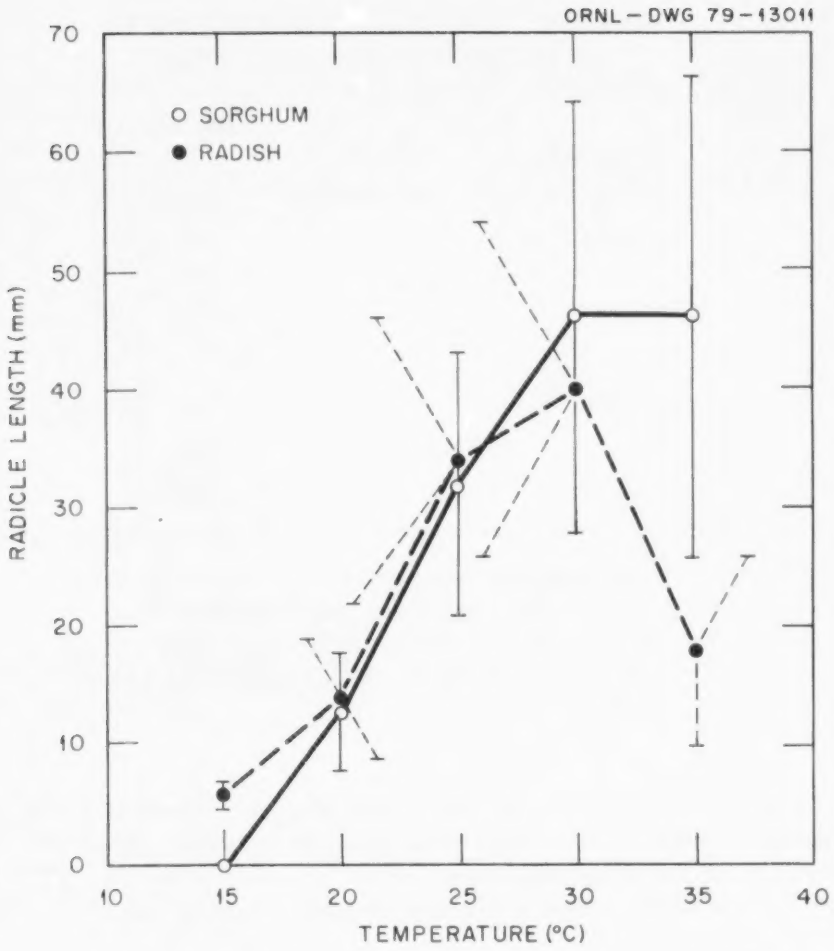

Figure 11. Radish and sorghum seeds were grown in germination chambers in the dark at various temperatures to quantify the effect of that parameter. Temperature ranged from 15 to 35°C in increments of 5°, and root length was measured after 48 h for radish and 72 h for sorghum.

Phytotoxicity of Acetic Acid

To determine the amount of dilution necessary to test the EP extracts without a confounding phytotoxic effect by the acetic acid used in the EP, we initially conducted short-term radish and sorghum root elongation tests at varying concentrations of acetic acid in water with the pH maintained at 5.0. The results of these tests (Table 24) indicated that an extract containing no more than 5 ml of acetic acid per l would be satisfactory for use in our tests with radish and sorghum. We assumed at that time that

TABLE 26. EFFECT OF HERBICIDES ON RADICLE LENGTH OF RADISH

Herbicide	Concentration* (mg/l)	Root length† (mm)	% Growth reduction
Dalapon	3.5	29 ± 17	12‡
	0	33 ± 17	—
2.4-D	0.2	21 ± 9	36‡
	0	33 ± 17	—
Diquat	50.0	29 ± 15	12‡
	0	33 ± 19	—
Dichlobenil	10.0	5 ± 3	85‡
	0	33 ± 19	—
Fenac	1.0	15 ± 8	57‡
	0	35 ± 19	—
Picloram	0.2	31 ± 16	11
	0	35 ± 19	—
Silvex	0.1	15 ± 7	57‡
	0	35 ± 19	—

*Concentrations were chosen as discussed in the text.

†Values given are mean ± S.D.

‡Significantly different from control at a 5% probability level.

this concentration would also be safe for wheat and soybean in the 2-week tests, and all further testing was conducted under this assumption. When time permitted, the same tests were conducted with wheat and soybean in longer-term (2 and 3 weeks, respectively) experiments, and we discovered that even 1 ml/l significantly reduced growth of wheat shoots and soybean roots (Figure 12). Growth reduction at this concentration, though statistically significant, was borderline in both cases and therefore probably had little or no influence on earlier tests with wheat and soybean in which this concentration was used. However, in cases in which acetic acid concentration exceeded 1 ml/l in the long-term tests, the results could be misleading. Results of the effects of plater's waste, metal processing waste, and retorted shale on soybean and wheat are questionable because of the concentration of acetic acid present in the extracts tested.

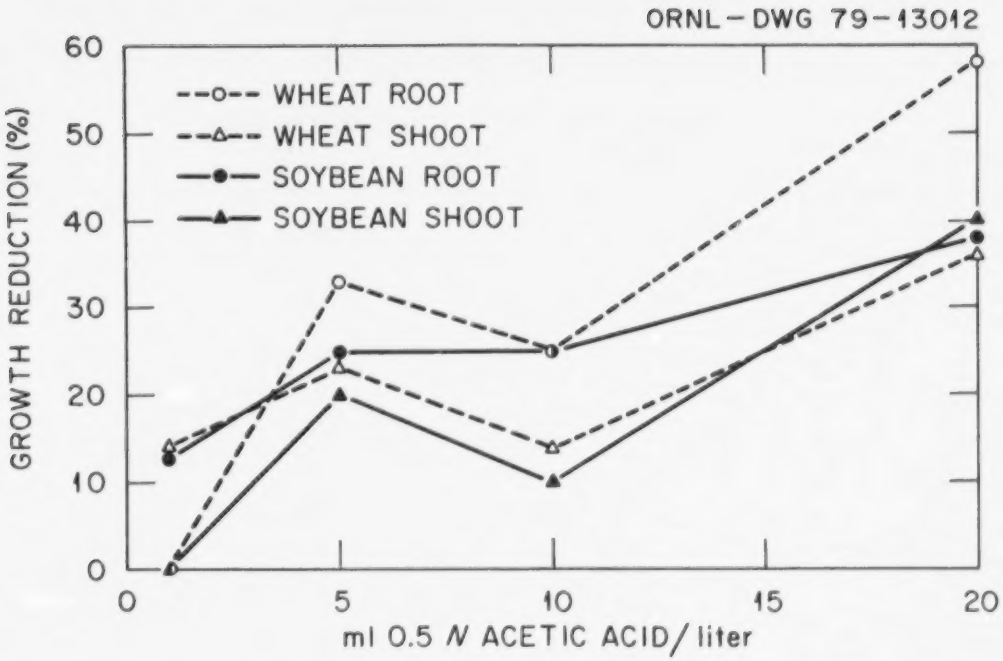

Figure 12. Effect of acetic acid on plant growth. Wheat and soybean seeds were planted in sand and grown under greenhouse conditions. Dilutions of 20, 10, 5, and 1 ml 0.5 N acetic acid per l solution were applied to wheat for 2 weeks and to soybean for 3 weeks. At harvest root and shoot lengths were measured (in cm) and treated and compared with controls for determination of percent growth reduction.

Because of the confounding effect that acetic acid has on phytotoxicity studies, especially long-term studies, we recommend that an extraction procedure be developed that does not use acetic acid.

SECTION 8

MUTAGENICITY

INTRODUCTION

Because the concept of toxicity includes unnatural genetic activity (including oncogenic, mutagenic, and teratogenic activity), EPA recognizes the need for rapid and effective methods for the detection of such activity in complex mixtures from the EP.

The bioassay protocol in this project is intended to serve as an indicator of the chronic hazards of mutagenicity and carcinogenicity. Because of systemic differences in reactivity to mutagenic substances, a battery of assays has been employed. The approach taken involves microbial assays, both eukaryotic and prokaryotic, which detect point mutation specifically and DNA damage generally.

Examination of a concentrated extract of the organic constituents of the EP extract (in addition to the extract itself) was considered advisable because: (1) the vast majority of known chemical mutagens are of organic constitution; (2) the concept of threshold is ill-defined for mutagens and carcinogens; and (3) the EP extract was anticipated to be low in organic character.

A further objective of this study was the validation of the yeast and repair assays with a series of known mutagens. Our goal was to determine the efficacy of metabolic activation in these two systems and their sensitivity to various mutagens.

METHODS

According to the definition of a waste as a hazard (in 43 FR 58960) the application of three assays for genetic activity would be required to delist a waste that is listed as hazardous because of mutagenic activities. The three tests specified are: (1) gene (point) mutations in bacteria; (2) gene mutations in eukaryotes, either in mammalian somatic cells in culture or in fungal microorganisms; (3) recombinogenic or repair-related phenomena. We have applied in these categories (1) the Salmonella/microsome assay, (2) the Saccharomyces $\underline{can}^r/\underline{his}^+$ dual assay, and (3) the Salmonella $\underline{uvrB}$ repair assay. We have had considerable previous experience applying the Salmonella and Saccharomyces mutation assays to the analysis of complex mixtures,

hence their inclusion. Because of the involvement of the uvrB mutation in the design of the Salmonella assay, the Salmonella repair assay was selected for group three.

The Salmonella/microsome assay utilizes a series of histidine-requiring mutants that revert after treatment with mutagens to the wild-type state (histidine-independent). Generalized testing is accomplished by use of three strains (TA1537, TA1538, and TA98) that detect frameshift mutagens and two strains (TA1535 and TA100) that detect base-pair substitution mutagens.

The Saccharomyces assay utilizes both a forward and a reverse mutation scheme. Forward mutation is detected by the inactivation of the arginine permease gene, leading to resistance to the toxic antimetabolite canavanine. Reverse mutation is monitored by use of a histidine auxotroph which reverts by base-pair substitution.

The Salmonella uvrB repair assay does not measure mutation per se, but rather DNA damage induced by chemical treatment. The test system employs paired, identical strains, except that one (TA1978) has normal DNA repair capabilities ($uvrB^+$) and one (TA1538) lacks a specific step ($uvrB^-$) in the enzyme pathways responsible for DNA repair. Preferential killing of the repair-deficient strain by the test substance implies that the material exerts its killing effect by reacting with the cells' DNA, and therefore may be mutagenic.

Full details of the experimental procedures for these assays are given in Appendices G, H, and I.

VALIDATION STUDY

Mutagens were chosen to include both directly and indirectly acting agents. Both alkylating and intercalating moieties are represented.

Yeast Mutation Assay

Table 27 gives dose-response results obtained with four directly acting mutagens. The threshold of sensitivity appears to be in the 0.1–1.0 μg/ml range. Extending the treatment period decreases the lower limit somewhat, but cytotoxicity increases disproportionately, increasing sampling error (not shown). Dose-response results for indirectly acting mutagens are given in Table 28. Threshold sensitivity values fall in the 10–50 μg range, although there is a great deal of compound-specific variability. It should also be noted that long incubation times are necessary to elicit a response.

Salmonella Repair Assay

Inactivation curves were constructed for the two indicator strains by use of selected mutagens. The results are expressed as LD_{50}s, shown in Table 29. TA1978 generally displays a shouldered semilogarithmic inactivation curve, typical of

TABLE 27. YEAST MUTATION ASSAY: DIRECTLY ACTING MUTAGENS

Mutagen* and concentration	% Survival at 3 h	$can^r/10^7$ survivors at 3 h	$HIS^+/10^7$ survivors at 3 h
Methyl methanesulfonate, %			
0.025	85	130	134
0.050	29	504	449
0.075	6	550	787
0.100	0	—	—
control	100	15	9
Ethyl methanesulfonate, %			
0.25	107	85	366
0.50	70	192	548
0.75	48	200	1,003
1.00	22	448	1,270
control	100	24	9
2(2-furyl)-3-(5-nitro-2-furyl) acrylamide, µg			
25	85	56	98
50	72	115	151
100	48	284	189
control	100	17	11
Nitroquinoline-N-oxide, µg			
0.10	91	446	178
0.50	73	1,811	897
1.0	18	3,955	2,659
control	100	30	10

*All mutagens were unactivated.

repair-competent strains. TA1538 gives roughly linear semilog plots. Generally, for a DNA-active compound the ratio of the percent survival figures (% survival TA1538/% survival TA1978) observed at the LD_{50} for TA1978 will be 0.1 or smaller (Table 30, upper)

Survival curves could not be obtained for several mutagens tested. Table 30 (lower) shows survival data for the highest dose tested. It appears that compounds requiring metabolic activation are only marginally active in the repair assay. The LD_{50}s

TABLE 28. YEAST MUTATION ASSAY: INDIRECTLY ACTING MUTAGENS

Mutagen and concentration	% Survival		$can^r/10^7$ survivors		$HIS^+/10^7$ survivors	
	3 h	24 h	3 h	24 h	3 h	24 h
2-Aminofluorene, μg						
With S-9						
100	95	97	103	173	28	47
50	98	102	74	81	20	24
25	102	99	12	19	11	10
control	100	100	9	7	8	8
Without S-9						
100	101	103	8	8	7	9
2-Aminoanthracene, μg						
With S-9						
100	80	67	23	156	8	39
50	94	82	19	70	6	17
25	101	96	14	21	11	9
control	100	100	16	13	9	11
Without S-9						
100	98	91	15	17	8	10
Benzo(a)pyrene, μg						
With S-9						
100	97	89	16	181	4	52
50	103	96	21	74	7	36
25	106	109	18	29	11	17
control	100	100	18	20	8	9
Without S-9						
100	93	82	17	19	9	12

(Continued)

(Table 28 continued)

Mutagen and concentration	% Survival		$can^r/10^7$ survivors		$HIS^+/10^7$ survivors	
	3 h	24 h	3 h	24 h	3 h	24 h
Dimethylnitrosamine, %						
With S-9						
4	100	97	105	215	107	236
2	99	104	103	224	92	181
1	103	111	74	153	79	148
control	100	100	28	31	7	12
Without S-9						
4	97	91	25	26	9	10
8-Nitroquinoline, µg						
With S-9						
1000	68	56	22	151	14	109
500	84	79	19	48	9	52
100	97	92	16	21	12	13
control	100	100	17	16	10	11
Without S-9	93	71	21	19	13	12

TABLE 29. LD_{50} VALUES FOR SALMONELLA REPAIR ASSAY

Mutagen	LD_{50} (μg) TA1978 uvrB$^+$	TA1538 uvrB$^-$
Methyl methanesulfonate, without S-9	6	0.8
Ethyl methanesulfonate, without S-9	71	15
Nitroquinoline-N-oxide, without S-9	20	0.15
2(2-furyl)-3-(5-nitro-2-furyl) acrylamide, without S-9	1.4	0.3
8-Nitroquinoline		
With S-9	110	12
Without S-9	> 1,000	130

for benzo(a)pyrene and dimethylnitrosamine with TA1538 were found to be 250 μg and 50 mg, respectively; other LD_{50}s were not determined.

Conclusions

The yeast mutation assay is suitable for the detection of directly and indirectly acting mutagens. Both alkylating and intercalating agents are detectable. The repair assay, however, seems effective only with directly acting mutagens. These are principally alkylating agents. Indirectly acting intercalating agents are marginally active (if at all) in the repair assay. The poor performance of the bacterial DNA repair assay emphasizes that positive control performance data must accompany test results for evaluation under RCRA.

ASSAY RESULTS

The aqueous EP extracts and their XAD-2 concentrates have been tested in the Salmonella/microsome assay, the Saccharomyces forward and reverse mutation assay, and the Salmonella repair assay. The assays were applied both with and without metabolic activation (both Ar- and phenobarbital (φB)-induced rat liver S-9 mix were used). The aqueous extracts were tested as received; the XAD-2 concentrates were taken up in 2 ml of dimethylsulfoxide, producing a 250-fold (v/v) concentration of the organic material in the aqueous extracts, with the exception of the arsenic-contaminated groundwater, whose concentration factor was 12.5-fold. The dye waste was tested only as the XAD-2 concentrate. For the sake of brevity, negative results are not shown.

TABLE 30. SALMONELLA REPAIR ASSAY: SURVIVAL AND SURVIVAL INDICES FOR SELECTED MUTAGENS

Mutagen and concentration	% Survival TA1978 uvrB$^+$	% Survival TA1538 uvrB$^-$	Survival index
Methyl methanesulfonate, 5 µl, no activation	47	0.13	0.003
Ethyl methanesulfonate, 50 µl no activation	64	5.9	0.092
Nitroquinoline-N-oxide, 1 µg, no activation	95	0.7	0.007
2(2-furyl)-3-(5-nitro-2-furyl) acrylamide, 5 µg, no activation	0.25	0.007	0.028
8-Nitroquinoline, 100 µg			
With activation	53	0.5	0.009
No activation	94	58	0.617
2-Aminofluorene, 1 mg			
With S-9	47	55	1.170
Without S-9	102	97	0.951
2-Aminoanthracene, 1 mg			
With S-9	74	50	0.676
Without S-9	92	86	0.935
Benzo(a)pyrene, 1 mg			
With S-9	103	9.6	0.093
Without S-9	88	120	1.364
Dimethylnitrosamine, 100 mg			
With S-9	77	28	0.364
Without S-9	105	108	1.029

*% survival TA1538/% survival TA1978.

Salmonella/Microsome Assay

The arsenic-contaminated groundwater was slightly mutagenic with the frameshift strain TA98, but only upon metabolic activation with Ar-induced S-9 mix (Table 31). The sample was not toxic at the concentrations tested. The XAD-2 concentrate of arsenic-contaminated waste was not mutagenic with the missense strain TA1535 (Table 32). It was mutagenic with the frameshift strains TA1537 and TA98 and with the highly sensitive TA100 strain. It did not require metabolic activation, and the addition of S-9 mix (Ar- or φB-induced) reduced the mutagenic activity. It was not toxic in the dose range tested. A dose-dependent response was elicited.

TABLE 31. SALMONELLA MUTATION: ARSENIC-CONTAMINATED GROUNDWATER EP EXTRACT

	Revertants/plate	
Volume (μl)	TA98	TA100
No activation		
control	59	152
0.025	NT*	NT
0.050	NT	NT
0.500	55	107
5.000	45	145
φB activation		
control	67	142
0.025	60	150
0.050	53	105
0.500	67	117
5.000	52	137
Ar activation		
control	67	142
0.025	87	193
0.050	87	151
0.500	70	190
5.000	96	141

*NT, not tested.

TABLE 32. SALMONELLA MUTATION: ARSENIC-CONTAMINATED GROUNDWATER XAD-2 CONCENTRATE

Volume (µl)	Revertants/plate			
	TA1535	TA1537	TA98*	TA100*
No activation				
control	12	10	59	152
2.5	NT†	NT	59	151
5.0	27	16	153	215
10	18	41	268	414
25	32	70	500	669
50	29	94	690	963
75	T‡	115	563	828
φB activation				
control			67	142
2.5			59	120
5.0			96	150
10	NT	NT	NT	226
25			79	288
50			206	414
75			NT	590
Ar activation				
control	6	7	67	142
2.5	NT	NT	82	155
5.0	11	15	97	162
10	15	27	NT	212
25	22	44	157	243
50	13	52	292	295
75	16	69	NT	508

*Average from two or three independent experiments.

†NT, not tested.

‡T, toxic.

None of the remaining wastes or their XAD-2 concentrates displayed mutagenic activity, with or without metabolic activation. Some cytotoxicity was encountered with the plater's waste and the fluidized bed residue.

In contrast, data from a pilot study of steam distillation extracts indicates a frameshifting mutagenic activity that is extractable from the metal processing waste (Table 33). Higher activity was observed in methylene chloride extracts than in cyclohexane extracts.

TABLE 33. SALMONELLA MUTATION: STEAM DISTILLATE FROM METAL PROCESSING WASTE

Strain*	Steam-distillate extract	Revertants/plate for given volume of steam distillate (µl)						
		0	10	25	50	75	100	150
TA98†	cyclohexane	31	41	54	61	67	44	—
	methylene chloride	31	55	48	74	97	85	107
TA100	cyclohexane	76	70	43	53	69	52	—
	methylene chloride	—	87	77	65	66	55	—

*Ar-induced S-9.

†Average of two independent experiments.

Saccharomyces Mutation Assay

The arsenic-contaminated groundwater sample was not mutagenic (Table 34). The XAD-2 concentrate of this sample was mutagenic without metabolic activation for a 24-h exposure (Table 34). A dose-dependent response was observed. Metabolic activation appeared to reduce the mutagenic potential of the XAD-2 concentrate. Neither of these test materials was toxic.

None of the remaining test materials possessed detectable mutagenic activity. No appreciable toxic effects were noted.

TABLE 34. YEAST MUTATION: ARSENIC-CONTAMINATED GROUNDWATER EP EXTRACT AND ITS XAD-2 CONCENTRATE

Concentration (μl)	% Survival				$can^r/10^7$ survivors				$HIS^+/10^7$ survivors			
	3 h		24 h		3 h		24 h		3 h		24 h	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation												
control	100	100	100	100	27	24	19	15	6	13	11	6
0.1	71		94		23		16		16		8	
1.0	84		81		20		19		8		8	
10	75	116	85	95	13	19	19	17	13	8	9	9
20		127		98		15		34		8		24
50		138		93		17		69		6		37
100	89	156	99	86	16	17	13	176	9	9	11	55
φB activation												
control	100	100	100	100	14	17	17	15	12	10	12	8
0.1	107		103		21		14		10		9	
1.0	59		87		27		21		10		10	
10	82	105	103	94	21	18	14	19	14	11	9	12
20		98		86		20		31		13		6
50		103		79		16		89		8		29
100	91	112	119	71	18	21	18	107	12	7	7	24
Ar activation												
control	100	100	100	100	17	15	21	14	9	9	11	6
0.1	97		94		18		20		11		9	
1.0	92		96		13		27		8		7	
10	87	107	91	108	21	12	22	13	12	10	10	8
20		111		122		25		41		11		9
50		122		91		13		74		5		17
100	88	90	103	82	24	13	22	118	11	8	13	38

Salmonella DNA Repair Assay

None of the materials displayed activity in this assay, either with or without metabolic activation. Nonspecific toxicity was moderate for most wastes.

DISCUSSION

Of the wastes discussed in this report, only the arsenic-contaminated groundwater possessed detectable mutagenic activity. For the purposes of bioassay, the mutagenic principal in the undiluted waste water is at the limit of resolution; XAD-2 concentration was necessary, however, to conclusively demonstrate mutagenic activity.

The response of the Salmonella/microsome assay to the arsenic-contaminated waste implies a frameshift mutation mechanism, which requires the addition or deletion of DNA base-pairs. Yeast results with this waste are similar, showing a moderate preponderance of induced forward mutation to can^r relative to induced reverion of the his base-pair substitution. This is typical for a response (by this system) to a frameshifting agent.

The arsenic-contaminated groundwater and its XAD-2 concentrate failed to elicit a response in the bacterial DNA repair assay. There are two key considerations: (1) the overall mutagenic potency of the waste is moderate, (2) validation studies have shown that the repair assay is particularly insensitive (although not unreactive) to frameshifting agents. Hence, it may not be significant to obtain a negative result in this context.

With regard to the negative results obtained with the remaining wastes, the aqueous extracts and XAD-2 concentrates of these materials are extremely deficient in organic character. The majority of organic mutagens are not detectable at the parts-per-billion level. Furthermore, inorganic mutagens (e.g., metals and metal complexes) which might be present are commonly not detectable by the bioassays in question.

Our experience has shown that biological testing — within the limits of the specific system used — can be carried out with complex organic materials, but perhaps only when coupled with the appropriate analytical separation schemes. An extrapolation to relative biohazard at this point would be, at least, premature. A number of precautions are given below.

As noted, aqueous materials will generally contain only low amounts of dissolved organics which may be biologically active. If it is the intent to determine whether mutagenic components are present in a given mixture, clearly, a concentration/fractionation scheme must be applied. However, the detection or perhaps the generation of mutagenic activity may well be a function of the chemical fractionation scheme utilized. The inability to recover specific chemical classes or the formation of artifacts by the treatment could well corrupt the results obtained in addition

to the possibility of an inability to detect the specific biological end point chosen. Along with the obvious bias that could accompany the choice of samples and their solubility or the time and method of storage, a number of biological discrepancies can also enter into the determinations. For example, concomitant bacterial toxicity can nullify any genetic damage assay that might be carried out. The dose-response relationship may not be linear, and some other method for a quantitative comparison may be mandatory. The choice of inducer for the liver enzymes involved can be wrong for selected compounds or mixtures. Furthermore, induction of metabolic enzymes of rat liver includes both activating and deactivating enzymes for potential mutagens. Results with mixtures requiring activation can be complex and different from those with pure organic compounds. Mutagenicity studies include not only proper metabolic activation systems but also appropriate quantitation of the metabolic enzymes (determined by titration studies) in the assays. Mutagenic analyses of complex mixtures of organic constituents activated with crude and complex enzyme homogenates require careful examination and cautious interpretation. The choice of strain in a reversion assay could be inappropriate for selected active components of a mixture; therefore, a battery of tests should be considered, including an assay for forward mutation.

Additionally, the applicability of the generally used Salmonella test to other genetic end points and the validation of the apparent correlation between mutagenicity and carcinogenicity still remains a point to be validated through significant fundamental research. The question of a correlation between mutagenic potency in the Salmonella assay and carcinogenic potency should be treated with caution. Again, the short-term assays chronically show negative results for certain substances with known biohazard, e.g., heavy metals and certain classes of organics. Similarly, compounds involved in or requiring cocarcinogenic phenomena would presumably go undetected. Recent studies do point to synergistic effects between compounds that may further complicate quantitative interpretation of results with complex mixtures.

As a prescreen to aid the investigators in ordering their priorities, short-term testing appears to be a valid approach with complex mixtures. Over-interpretation at this stage of research, especially with respect to relative hazard or negative results, should be avoided.

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APPENDIX A

EXTRACTION PROCEDURE (43 FR 58956)

(A) EQUIPMENT

(I) An agitator which, while preventing stratification of sample and extraction fluid, also insures that all sample surfaces are continuously brought into contact with well-mixed extraction fluid.

(II) Equipment suitable for maintaining the pH of the extraction medium at a selected value.

(B) PROCEDURE

(I) Take a representative sample (minimum size 100 g) of the waste to be tested. Separate sample into liquid and solid phases. The solid phase is defined as that fraction which does not pass through a 0.45-μm filter medium under the influence of either pressure, vacuum, or centrifugal force. Reserve the liquid fraction under refrigeration (1–5°C) for further use.

(II) The solid portion of the sample, resulting from the separation procedure above or the waste itself (if it is already dry), shall be prepared either by grinding to pass through a 9.5-mm (3/8 in.) standard sieve or by subjecting it to the structural integrity procedure.

(III) Add the solid material from paragraph II to 16 times its weight of deionized water. This water should include any water used during transfer operations. Begin agitation and extract the solid for 24 ± 0.5 h. Adjust the solution to pH 5 and maintain that pH during the course of the extraction using 0.5 N acetic acid. If more than 4 ml of acid for each g of solid would be required to maintain the pH at 5, then once 4 ml per g of solid has been added, complete the 24-h extraction without adding any additional acid. Maintain the sample between 20–40°C during extraction.

(IV) At the end of the 24-h extraction period, separate the sample into solid and liquid phases as in paragraph I. Adjust the liquid phase with deionized water so that its volume is 20 times that occupied by a quantity of water at 4°C equal in weight to the initial sample of solid (e.g., for an initial sample of 1 g, dilute to 20 ml). Combine this liquid with the original liquid phase of the waste. This combined liquid, including precipitate which later forms from it, is the Extraction Procedure extract.

APPENDIX B

EXTRACTOR

APPARATUS

Apparatus used in the extraction is shown in Figures B-1 to B-11. Commercial apparatus utilized included: IEC constant-temperature centrifuge; Millipore filtration assembly (cat. no. YY42-142-00, filter cat. no. HAWP-142-50); Chemtrix pH controller (cat. no. 45A); Masterflex tubing pump and pump head (cat. no. 7045-10 and 7013-00); Cole-Palmer stirring motor (cat. no. 4558).

GLASSWARE CLEANING AND REAGENTS

Prior to extraction, all glassware and extractors were cleaned with detergent followed by dilute nitric acid, then thoroughly rinsed with autoclaved deionized water from a Millipore Milli-Q water purification system. The deionized water was then autoclaved (15 psig, 127°C, 20 min) before use in cleaning and extracting. In all cases, reagent-grade chemicals were used.

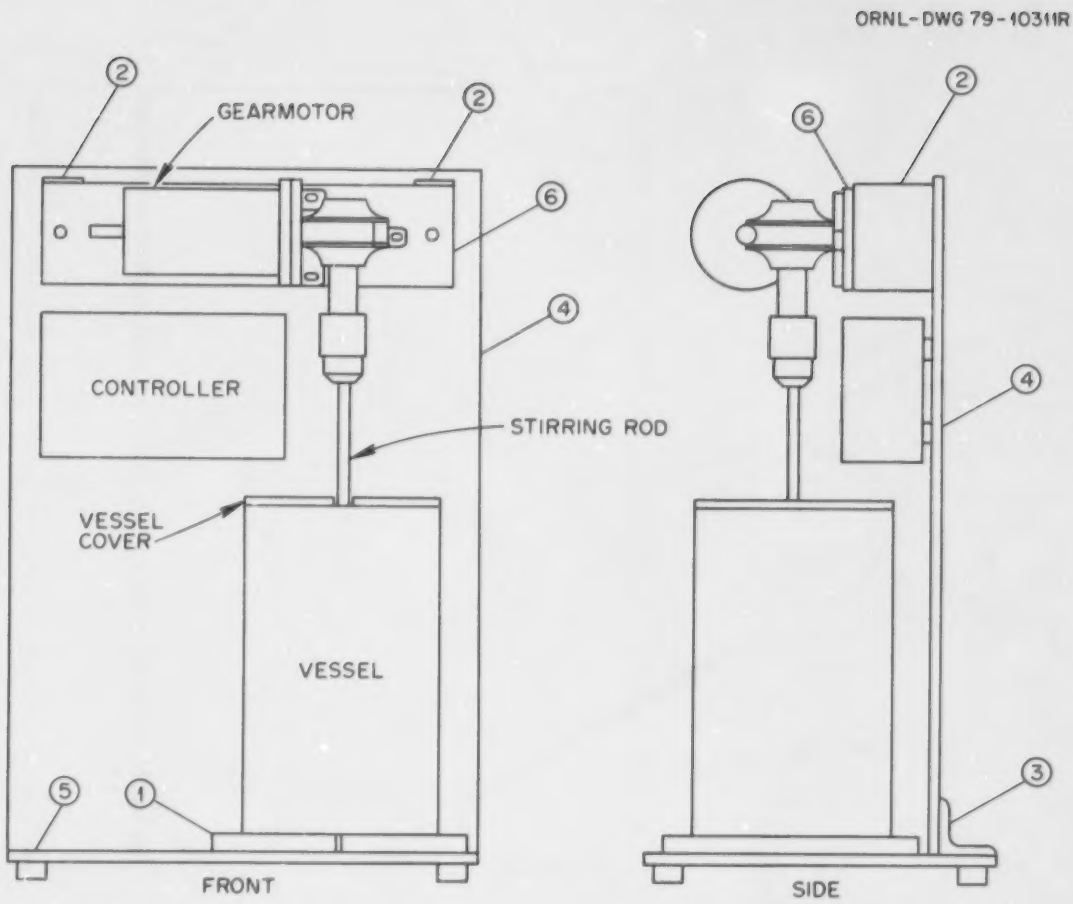


Figure B-1. Extraction apparatus.

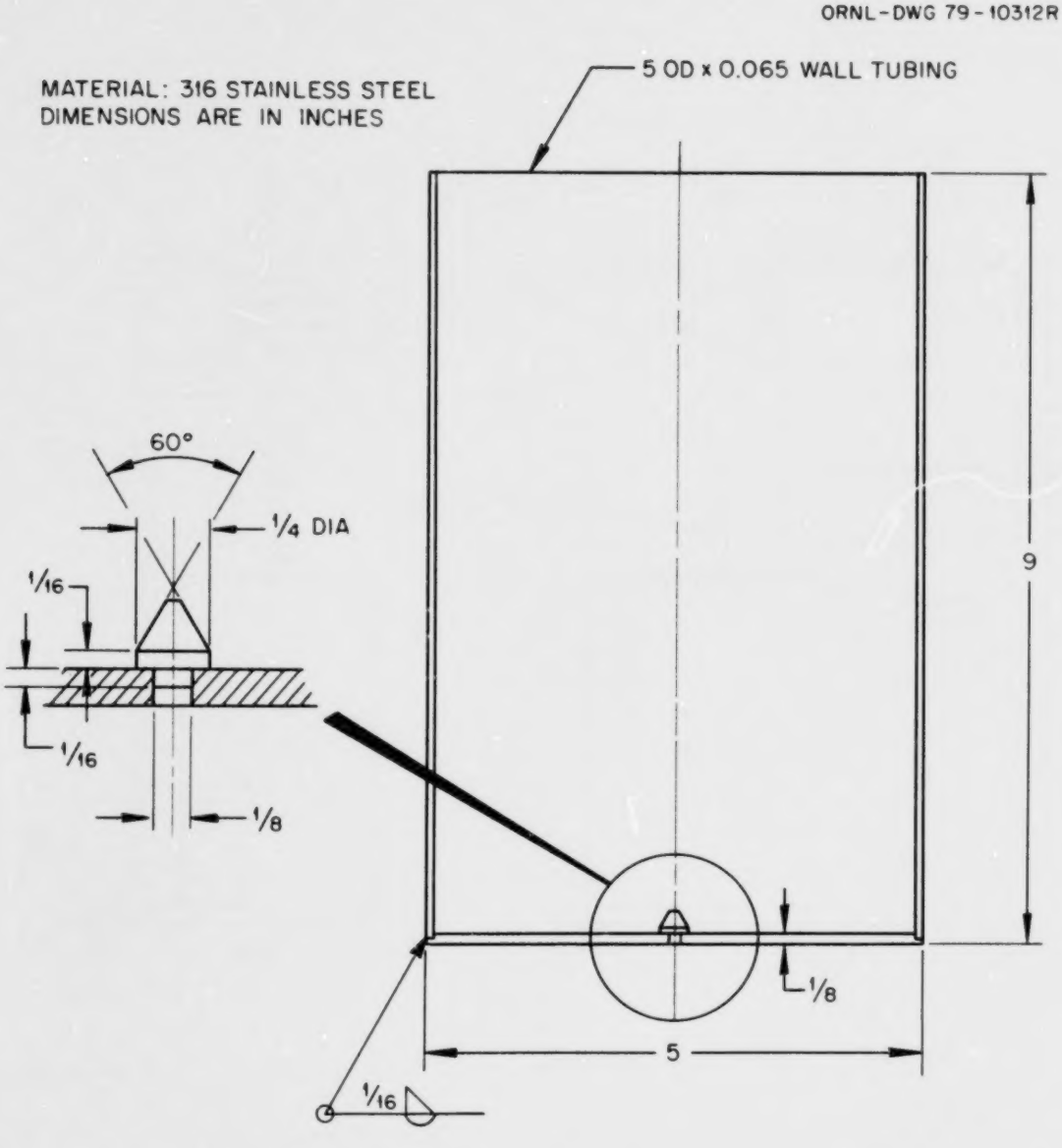


Figure B-2. Extracting vessel.

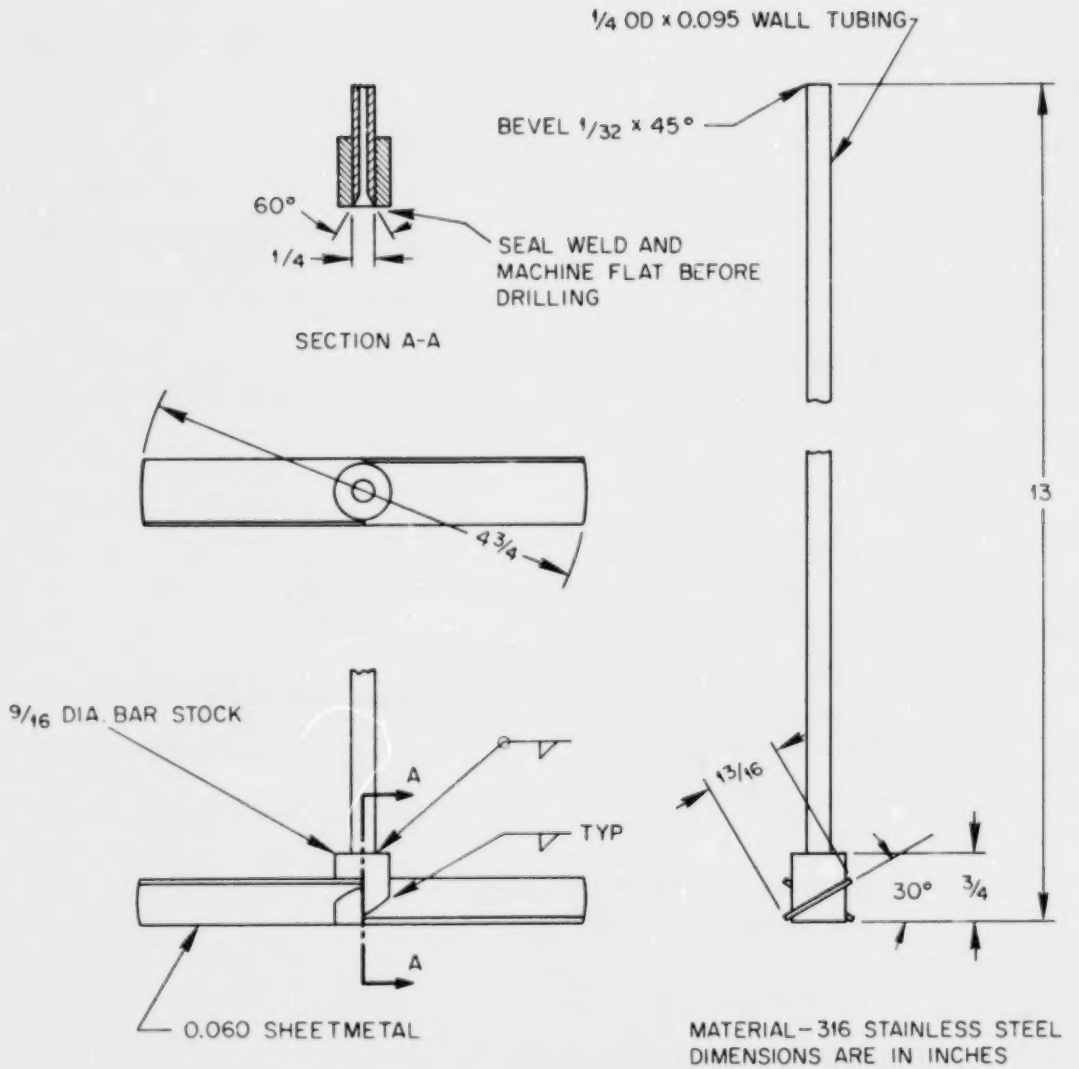


Figure B-3. Stirring rod.

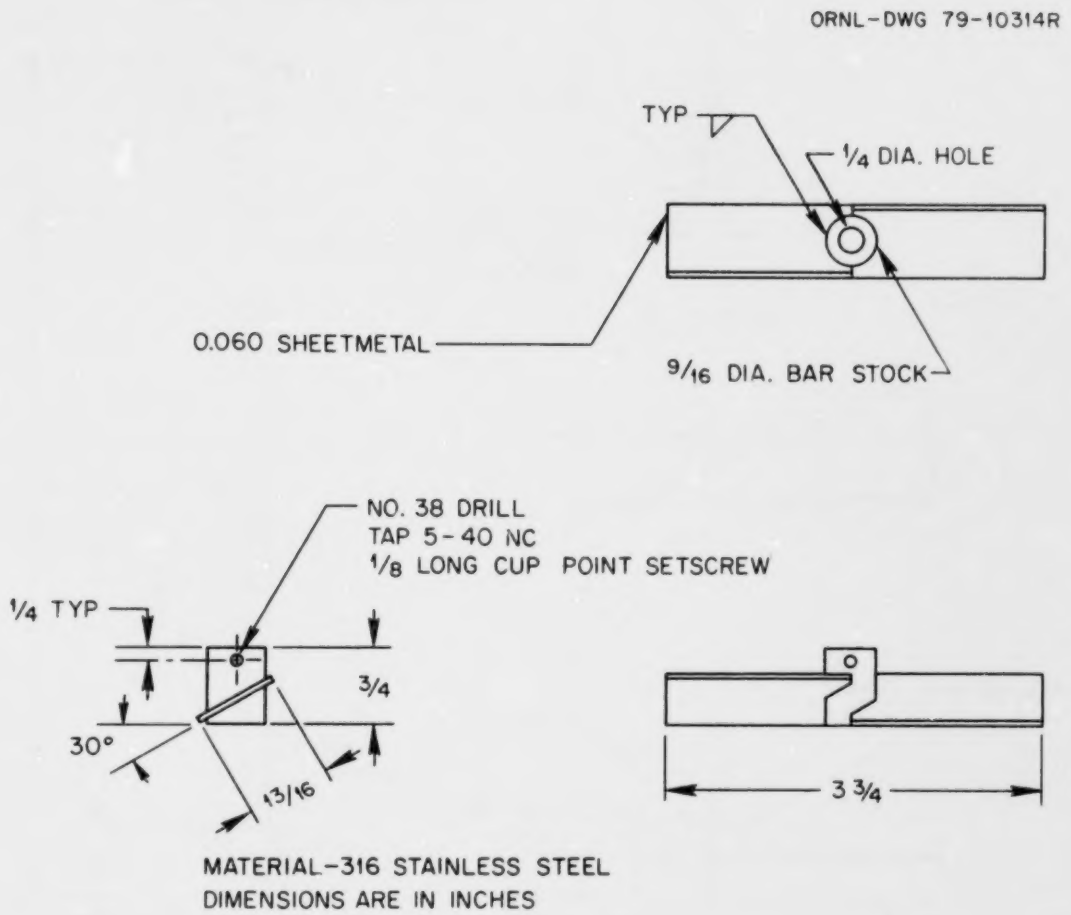


Figure B-4. Upper stirring blade.

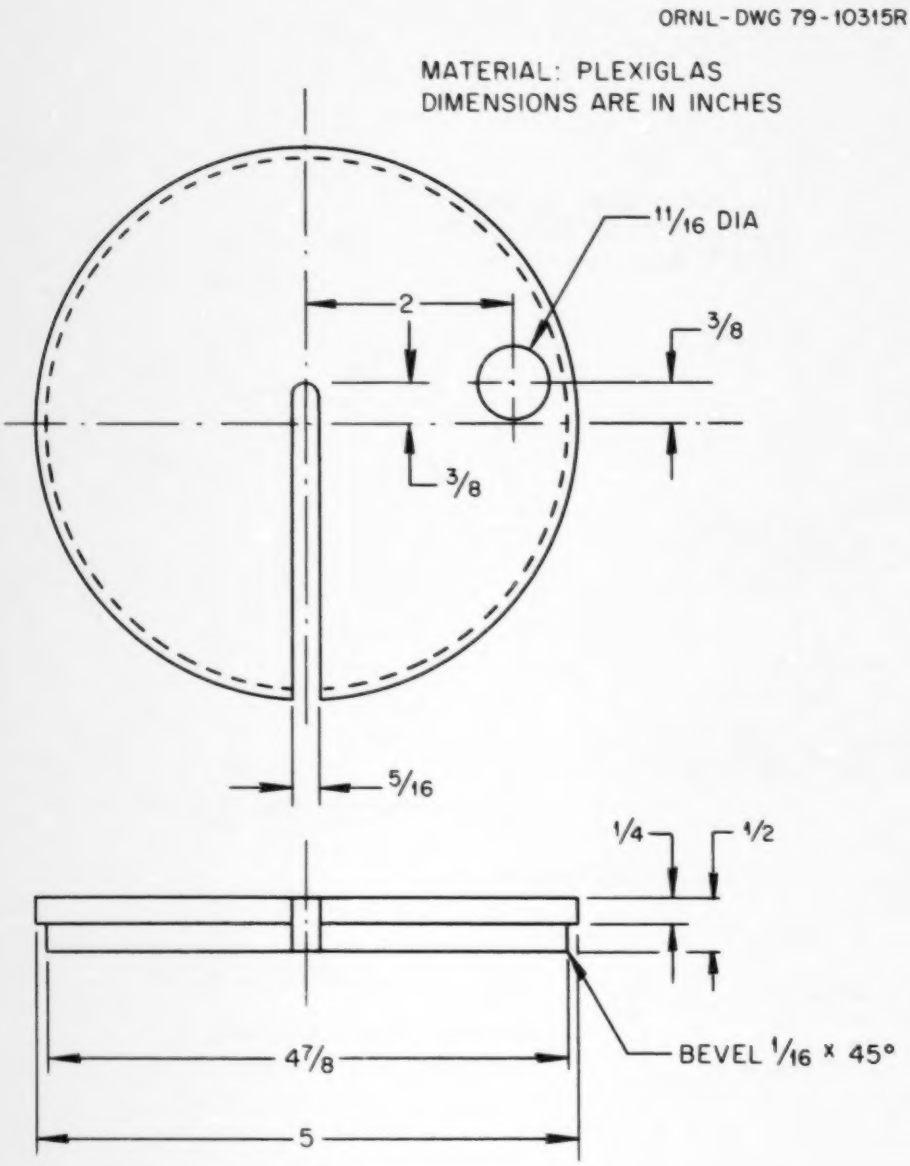


Figure B-5. Vessel cover.

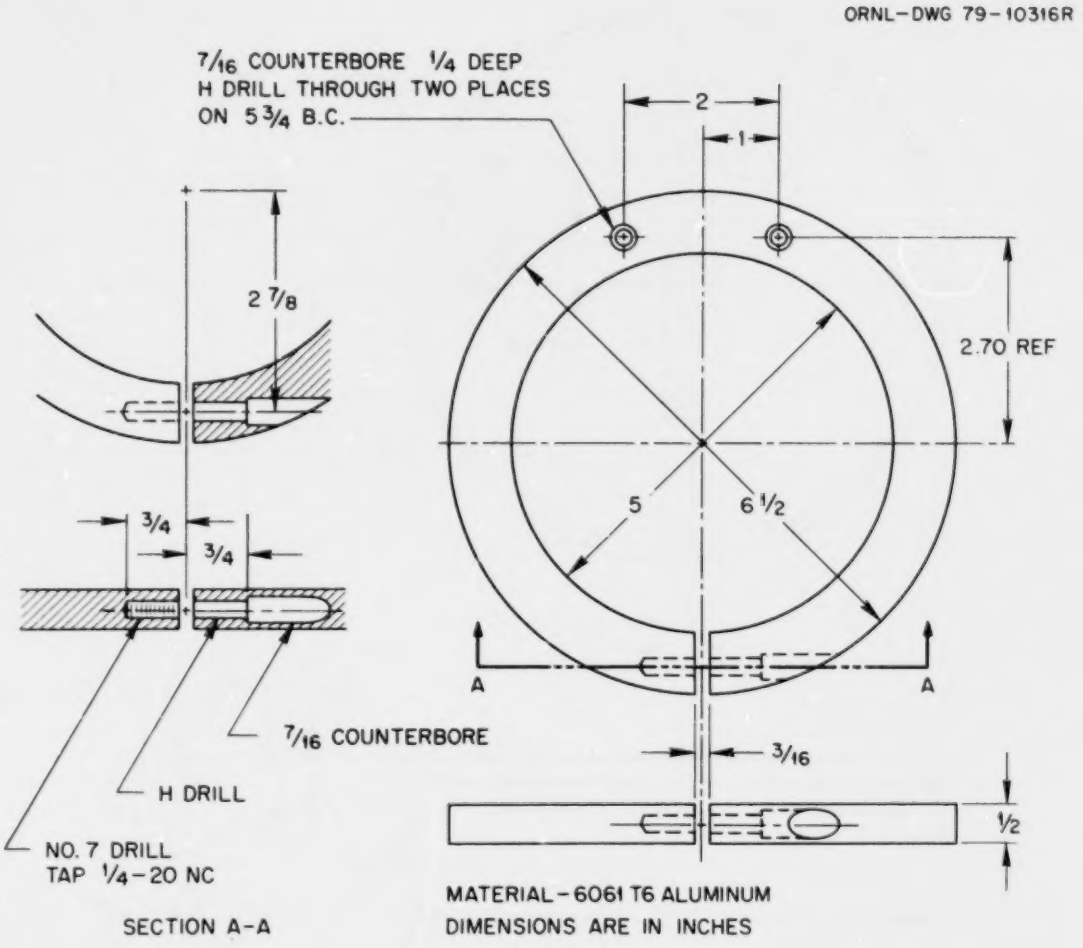
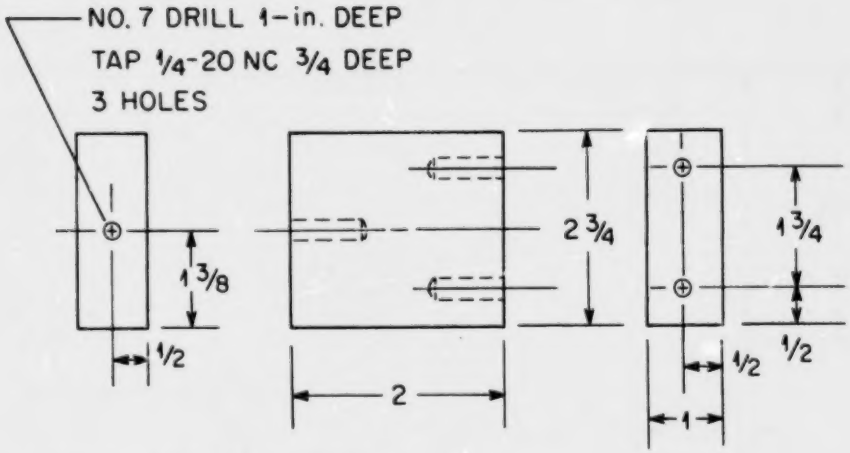


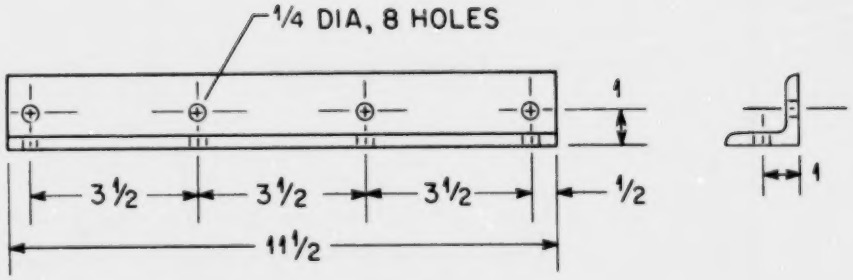
Figure B-6. Part No. 1.

ORNL-DWG 79-10317R



MATERIAL 6061T6 ALUMINUM
MAKE 2

PART NO. 2



MATERIAL - 1 1/2 x 1 1/2 x 1/4
ALUMINUM ANGLE

PART NO. 3

Figure B-7. Parts No. 2 and 3.

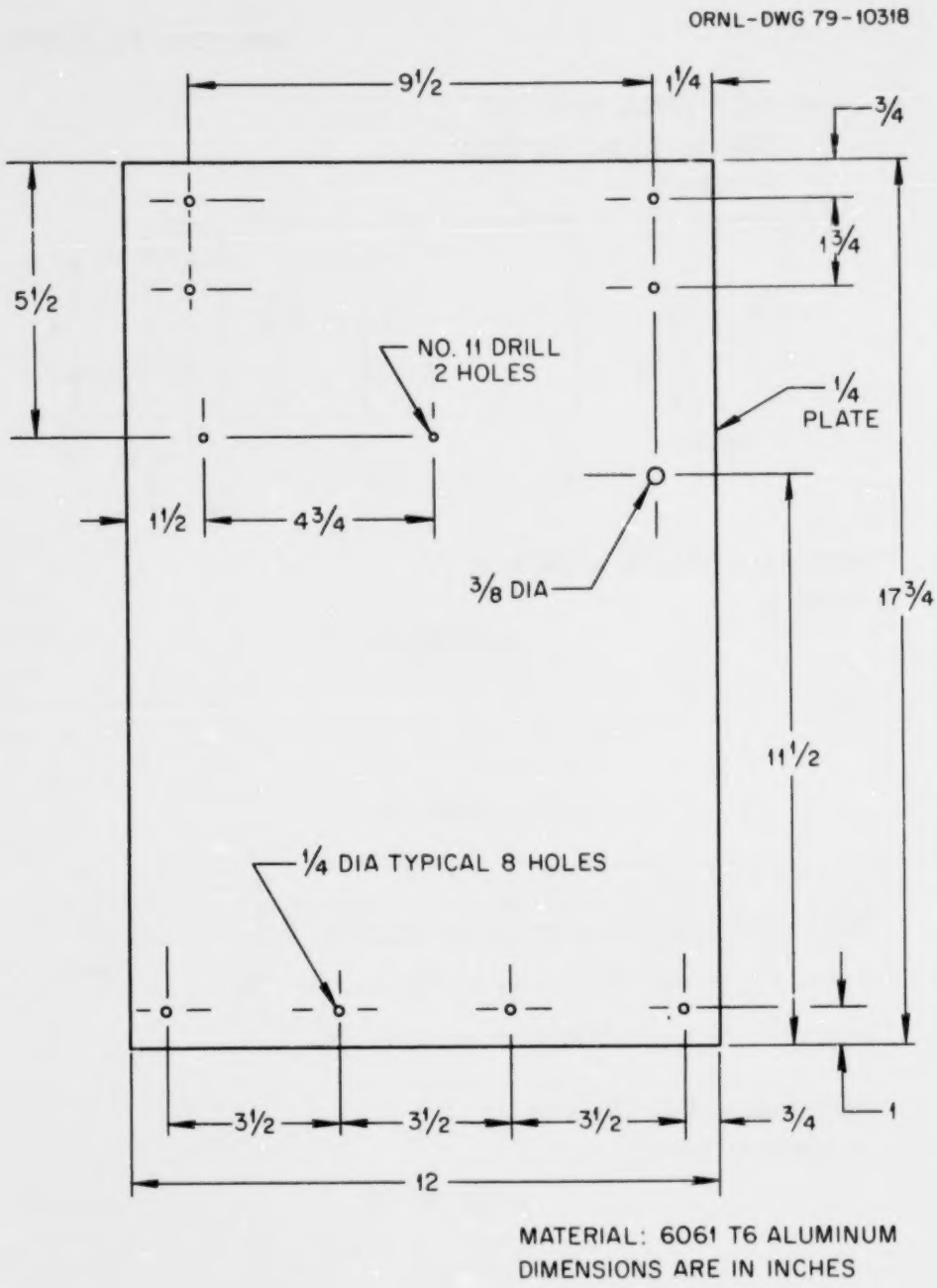


Figure B-8. Part No. 4.

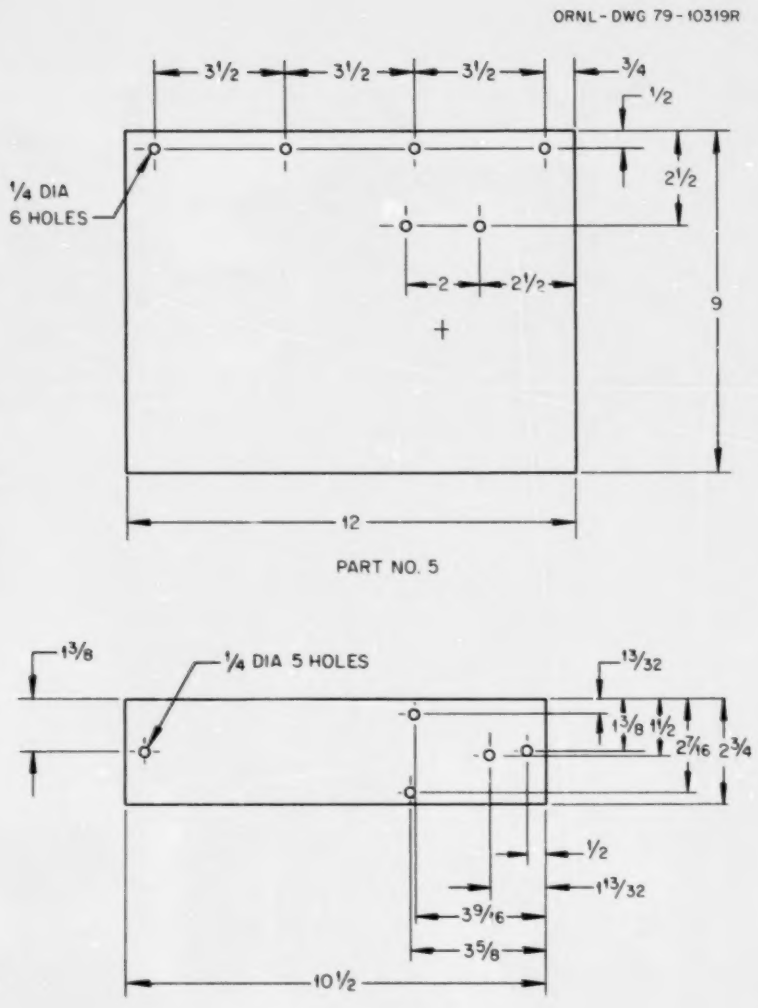


Figure B-9. Parts No. 5 and 6.

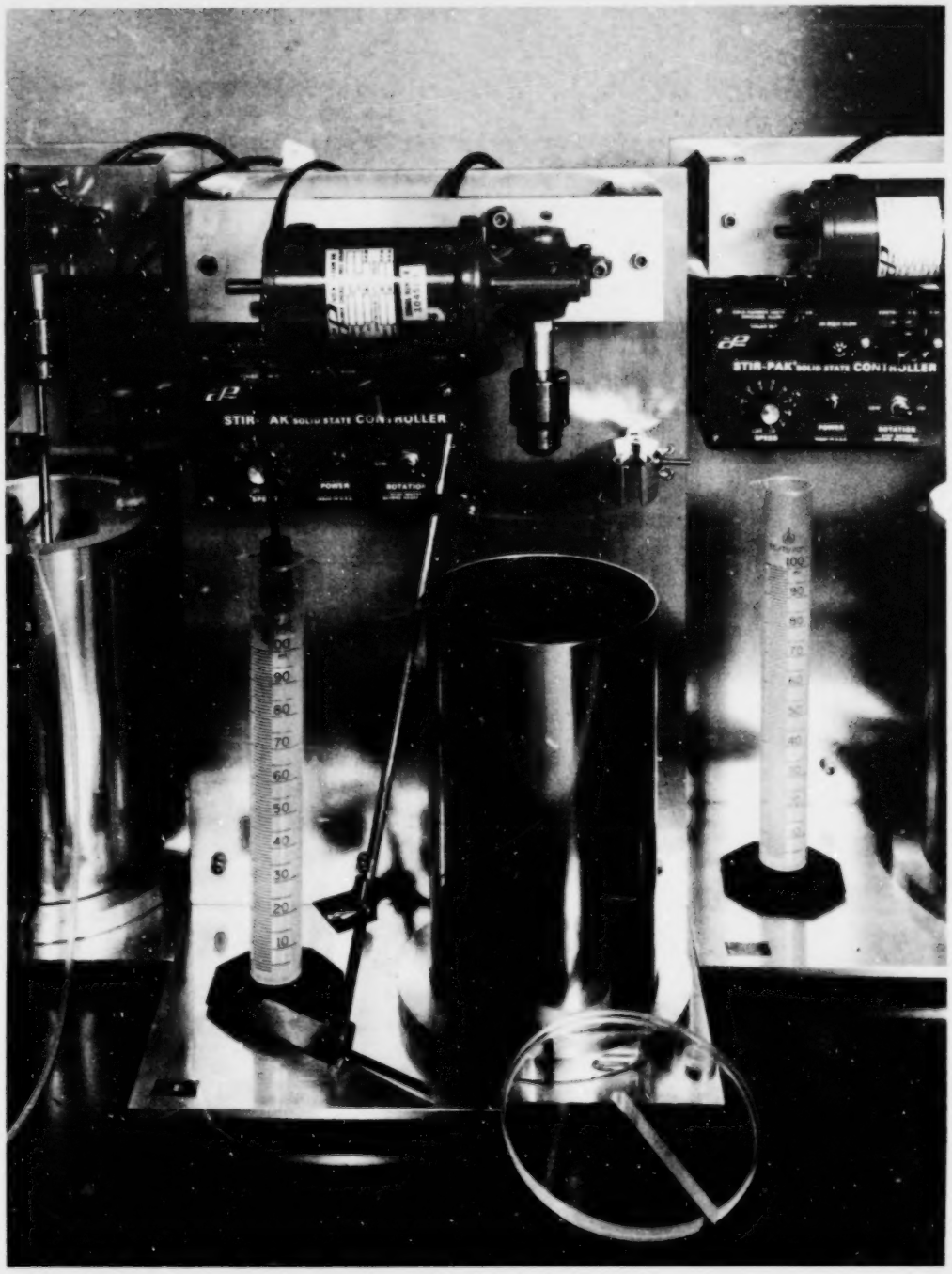


Figure B-10. Extraction apparatus.

Figure B-11. Laboratory set-up for EP.

APPENDIX C

EXPERIMENTAL PROTOCOLS FOR CHEMISTRY

The procedures employed for the chemical analysis of the EP extracts of solid wastes are described below. Specific GC conditions are given in Table C-1. Carrier gas flow rates should be optimized for the particular instrument used.

TABLE C-1. GC CONDITIONS FOR ANALYSIS OF ORGANIC CONCENTRATE FRACTIONS

Fraction	GC column	Column temperature (°C)	Detector*
Polar	10 ft x 0.125 in. O.D., glass, 4.8% Dexsil 400 on 100/120 Gas Chrom Q	100–320 at 4°/min	FID
PCB/pesticide	3 ft x 0.25 in. O.D., glass, 1.5% OV-101 on 100/120 Gas Chrom Q	130–230 at 2°/min	ECD
Diaromatic, polyaromatic, and heteroaromatic	10 ft x 0.125 in. O.D., glass, 4.8% Dexsil 400 on 100/120 Gas Chrom Q	100–320 at 4°/min	FID
Volatile organics	50 m glass capillary, 0.2% diethylene glycol succinate	-70†–175‡ at 1°/min†	FID
Polar,§ and o-nitroaniline	42 m glass capillary, 0.25% Carbowax 20-M	110–200 at 2°/min	FID

*FID, flame ionization detector; ECD, electron capture detector.

†Column temperature during thermal desorption of Tenax cartridge.

‡Column temperature during analysis.

§Initial work only. Dexsil 400 packed column used for most of analyses.

PREPARATION OF ORGANIC CONCENTRATE

This procedure is based on work by Junk et al.[1] and Yamasaki and Ames.[2] A 500-ml aliquot of the EP extract is adjusted to pH 6.8 with Na_3PO_4 and to 20 mmho/cm conductance with NaCl. This preparation is then passed through 4 g of XAD-2 (Isolab, Inc.) at a flow rate of 1.2 ml/min. The column is rinsed with 15–20 ml deionized water, and then adsorbed constituents are eluted with 10 ml redistilled acetone. The acetone eluate is evaporated under N_2 to dryness and dissolved in 10 ml cyclohexane for further fractionation. This procedure is carried out in quadruplicate to provide duplicate samples for chemical analysis and mutagenicity testing.

FRACTIONATION OF ORGANIC CONCENTRATES

The cyclohexane sample resulting from the XAD-2 procedure is fractionated by column chromatography. It is passed through 10 g of Florisil, eluted with 150 ml of 6/1 hexane/benzene, and passed through 20 g of neutral, activity III (4% moisture) alumina with 150 ml hexane followed by 150 ml 6/1 hexane/benzene, 160 ml 2/1 hexane/benzene, and 150 ml of acetone. Each fraction is concentrated to 1 ml with dry, flowing nitrogen under reduced temperature and pressure.

ANALYSIS OF PCB/PESTICIDE FRACTION

Five microliters of the hexane eluate of the alumina column are analyzed by packed-column GC with electron capture detection and temperature programming. External standards are used for calibration.

ANALYSIS OF PARAFFIN/MONOAROMATIC/DIAROMATIC FRACTION

Five microliters of the hexane eluate of the alumina column are analyzed by packed-column GC by flame ionization detection and external standardization.

ANALYSIS OF DIAROMATIC AND POLYAROMATIC FRACTION

Five microliters of the 6/1 hexane/benzene and 5 µl of the 2/1 hexane/benzene fractions are analyzed by the same GC conditions as noted immediately above.

ANALYSIS OF OTHER HALOGENATED OR POLAR ORGANICS

A scan for the presence of other halogenated compounds is carried out for each of the other benzene fraction concentrates obtained from the alumina step in the fractionation scheme. Five microliters from each fraction are injected into a gas chromatograph equipped with a 10-ft OV-101 column at 180°C and an electron-capture detector. Further scans are set up for detection of polar compounds retained by the Florisil and alumina columns during fractionation. These columns are further eluted with 150 ml redistilled acetone, which is then concentrated to 1 ml with dry, flowing nitrogen. Five microliters of this acetone concentrate are injected into a gas chromatograph equipped with a 42-m glass capillary column (0.25% Carbowax 20-M) and temperature-programmed from 110 to 200°C at 2°/min. Flame ionization detection is used. Later studies employed the packed Dexsil 400 column.

ANALYSIS OF o-NITROANILINE

A 50-ml aliquot of the arsenic-contaminated groundwater is extracted four times with 20 ml of methylene chloride, and the combined organic layers are concentrated to 1 ml by dry, flowing nitrogen under reduced temperature and pressure. A 0.4-µl sample is analyzed by GC on the Carbowax 20-M capillary column as described immediately above.

ANALYSIS OF VOLATILE ORGANICS

The procedure is a modified version of that by Grob.[3] The aqueous sample (5 ml) is purged with N_2 at 100 ml/min for 45 min into a 1 cm x 1 mm precolumn packed with 2 mg Tenax (60–80 mesh) and 1 mg Florisil, in series. The volatiles are desorbed from the precolumn in the injector (250°C) of a gas chromatograph with the analytical column held at -70°C. The analytical column is a 50-m glass capillary coated with 0.2% diethylene glycol succinate. The pollutants are detected by flame ionization during a temperature-programmed run from 0 to 175°C at 1°C/min with a 32-min final hold. Quantification is carried out with external standards treated similarly. Samples are analyzed in duplicate.

ANALYSIS OF DISSOLVED METALS

Duplicate aliquots of the EP extracts are directly analyzed for metals by flameless graphite furnace AAS. Calibration is conducted with external standards, and one sample aliquot is spiked to check recoveries.

Aliquots of EP extracts for Hg determination are preserved by addition to a nitric acid/dichromate solution immediately after generation and are worked up for cold vapor flameless AAS analysis by a published procedure.[4]

Selenium is chelated and extracted into toluene by a published procedure[5] prior to analysis by flameless graphite furnace AAS.

Arsenic is determined by an arsine accumulation–helium glow detector procedure.[6]

REFERENCES FOR APPENDIX C

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2. Yamasaki, E., and B. N. Ames. Concentration of Mutagens from Urine by Adsorption with the Non-polar Resin XAD-2: Cigarette Smokers Have Mutagenic Urine. Proc. Natl. Acad. Sci. USA, 74: 3555, 1977.
3. Grob, K. Organic Substances in Potable Water and Its Precursor: Part I. Methods for Their Determination by Gas-Liquid Chromatography. J. Chromatogr., 84: 255, 1973.

4. Feldman, C. Perchloric Acid Procedure for Wet-ashing Organics for the Determination of Mercury (and Other Metals). Anal. Chem., 46: 1606, 1974.
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6. Feldman, C. Improvements in the Arsine Accumulation–Helium Glow Detector Procedure for Determining Traces of Arsenic. Anal. Chem., 51: 664, 1979.

APPENDIX D

MATERIALS AND METHODS FOR THE AQUATIC TOXICITY SCREENING TESTS

The tests, in order of occurrence, were: (1) A preliminary 48-h acute toxicity determination; a few test concentrations over a wide range. (2) A definitive 48-h acute toxicity test; test concentrations over a narrower range than in (1). (3) A 28-day life-cycle, chronic toxicity test; two dilutions (1:100 and 1:1000) of the extract. (4) A final 48-h LC_{50} determination (this determined if the toxicity of the extract had changed during the 28-day life-cycle test).

ACUTE TOXICITY TESTS

Laboratory-cultured, first-instar Daphnia magna, less than 24 h old, were the test animals. Five organisms were exposed to 80 ml of extract solution in covered 100-ml glass beakers. Temperature was maintained at 20 ± 0.5°C in an environmental chamber with alternate light/dark periods of 12 h each. The dilution water used was well water with a pH of 7.8, an alkalinity of 119 mg/l, and a hardness of 140 mg/l. The extracts were neutralized to pH 7.0 with NaOH. The pH of the extract dilutions was measured at the beginning and conclusion of each test.

Serial geometric dilutions with well water were made for each extract. The concentration of each extract solution was 60% of the preceding one. The range of dilutions was selected to bracket 48-h LC_{50} values obtained from preliminary toxicity tests. Controls consisted of animals exposed to: (1) well water without extract, or (2) neutralized hydrochloric or acetic acid equal to the highest concentration of acid used in the acute toxicity tests. All tests were done in triplicate. The range of dilutions was selected to bracket 48-h LC_{50} values predicted from preliminary toxicity determinations. Additional control beakers were included containing a concentration of neutralized acetic acid equal to the highest concentration used in the acute toxicity tests. Control beakers of dilution water without added extract were also included. Values for 48-h LC_{50} and 95% fiducial intervals were obtained by computerized PROBIT analytical procedures.

CHRONIC TOXICITY TESTS

The test animals were first-instar D. magna less than 24 h old. They were exposed individually to 50 ml of either 1:100 or 1:1000 dilution of the extract in covered 100-ml beakers. Temperature and lighting conditions were the same as for the acute tests. In each test, 40 animals were used as follows: 10 were exposed to each

test dilution, 10 to well water only, and 10 to neutralized acetic or hydrochloric acid at the same concentration as used in the 1:100 dilution (acid was used in the EP, therefore an appropriate control was necessary). The animals were transferred to freshly prepared test solutions three times a week, and at those times they were fed 2 mg of prepared trout chow. At the time of transfer, the number of young and number of broods present in each beaker were counted. The pH of the test solutions was measured at the beginning and end of each test. The tests lasted 28 days, or less if all animals had died before that time.

APPENDIX E

RADICLE LENGTH ASSAY

Two levels of tests were completed on 17 solid waste extracts by use of the EP. Of these, 11 were from coal processing plants and 6 were from various types of industrial plants. One test was run on arsenic-contaminated groundwater taken from a well near a disposal site. Level one studies consisted of a root elongation bioassay of radish (Raphanus sativus L. c.v. Early Scarlet Globe) and sorghum (Sorghum vulgare var. saccharatum c.v. Sugar Drip) seeds. In previous tests, seeds were germinated in petri dishes and root (radicle) lengths of treatment and controls were compared. However, the time required to measure root lengths was so great due to their coiled growth pattern that it was not practical to use enough seeds for good statistical comparisons. Therefore, we developed special vertical germination chambers which took advantage of the geotropic growth response of plants, resulting in straight hypocotyl growth and a tenfold reduction in measurement time.

One approach to reduce variability was to sieve seeds to separate them into size categories. U.S.A. standard testing sieves numbers 8, 10, and 12 with openings (in mm) of 2.36, 2.00, and 1.70, respectively, were used for separation. Within a test only one seed size was used for controls and test dilutions. Although 200 seeds were used for each treatment, only 150 seeds were actually measured. The excess allowed for exclusion of nongerminating seeds and for radicles which were less than 5 mm long.

The germination chambers were constructed of 3-mm-thick Plexiglas (Figure E-1) with inside dimensions of 10 cm high x 1.5 cm wide x 71 cm long. The size of the chambers was determined by the size of the incubator in which they were to be used. Chambers were mounted on a Plexiglas base support. Two pieces of 3-mm-thick Plexiglas were cut to an appropriate size to fit inside a chamber but extended above the chamber sides about 3 cm for convenience in handling. One hundred depressions (drilled with an electric drill and bit) spaced at 2-cm intervals in staggered rows 2 cm apart across one of the Plexiglas sheets served as seed counters, seed spacers, and to help hold the seeds in place. Seeds were placed on the Plexiglas sheet and brushed into the depressions. A piece of blotter paper was saturated with the solution to be tested and pressed firmly against the seeds until impressions were seen. Additional test solution, up to a total of 100 ml for 48-h tests and 125 ml for 72-h tests, was added to the germination chambers. We recommend that initially the blotter paper be saturated with the extract in a flat tray rather than standing on edge in the chambers, since standing on edge could result in differential movement of chemicals up the paper causing chromatographic separation and variable doses to seeds at different positions. The second Plexiglas sheet was positioned so that the seeds and blotter paper were sandwiched in between the two sheets of Plexiglas, which were than taped securely on the sides and top and placed vertically into the chamber. A Plexiglas lid was placed on

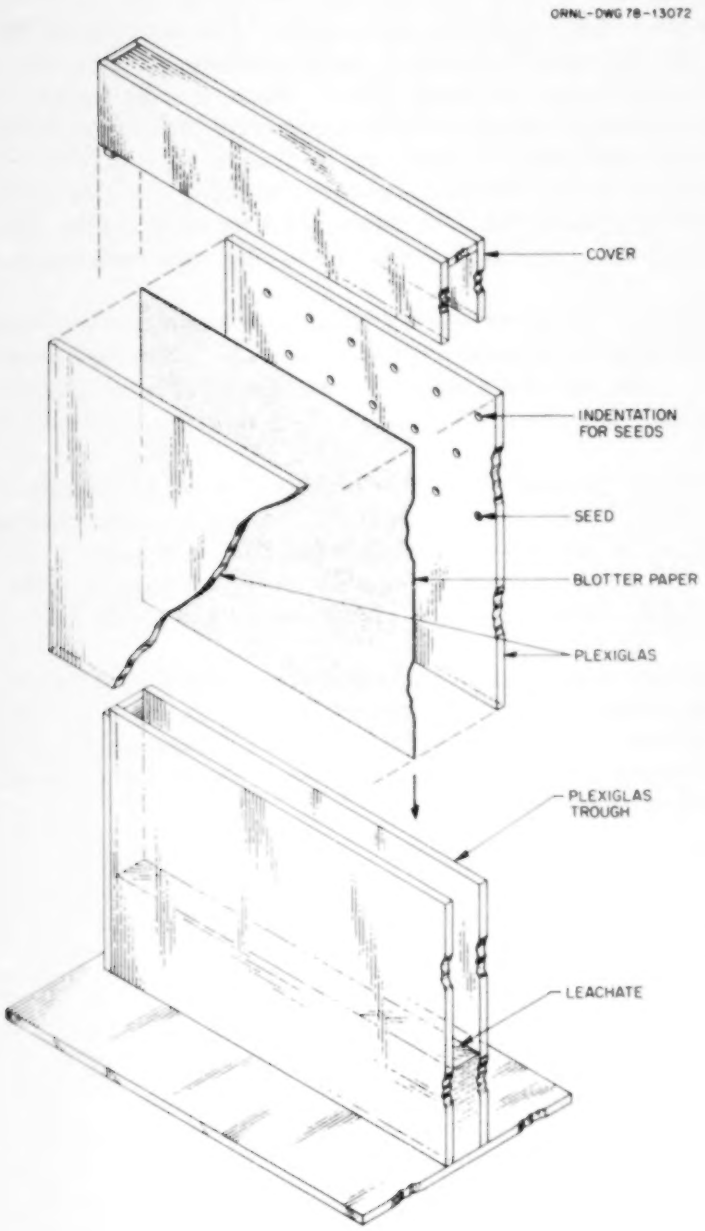

Figure E-1. Germination chamber.

top to reduce evaporation. The entire apparatus was then placed in an unlighted incubator set at 25°C. A small fan was installed in the growth chamber to exhaust volatiles after some tests had already been made. The fan affected the chamber temperature, thus thermostat adjustments were necessary. During this period of adjustment, tests continued, to avoid delay. Since controls are run with each test, we were not concerned about effects of these temperature differences between tests. We used two chambers (200 seeds) for each test solution or for each concentration of a particular solution and two chambers containing distilled water as controls. After a predetermined time period (48 h for radish, 72 h for sorghum) the chambers were removed from the incubator and the root lengths were measured with calipers.

For cleaning, the chambers were filled with an appropriate cleaning solution (0.1 N HCl) and allowed to stand until their next use, when they were rinsed with distilled water. The rest of the apparatus was washed with two pipette washers, one containing 1 N HCl and one connected to a distilled water supply for rinsing.

Acetic acid was used in the EP to maintain the pH of the extract at approximately pH 5.0. Since acetic acid is toxic to plants, the highest concentration of extract used in the root elongation test was the concentration having less than 5.5 ml/l of 0.5 N acetic acid. In preliminary tests this and higher concentrations of the organic acid were toxic to radish, sorghum, wheat, and soybean seeds (Table 23).

The material referred to as the arsenic-contaminated groundwater sample was not carried through the EP, but was diluted directly from the original solution for the root elongation tests. Since this particular waste was extremely toxic (based on an initial test with radish seeds) and safety problems were not yet resolved, further study (greenhouse testing) was not undertaken.

APPENDIX F

SEEDLING GROWTH ASSAY

The long-term seedling growth studies were conducted with wheat (Triticum aestivum c.v. Bear) and soybean (Glycine max c.v. Centennial). Soybean plants were grown in 1 l of sand and wheat in 1.5 l. To 1 l of sand (sand which passes through a 25-mesh sieve) was added 350 ml of a 10% concentration of a solution containing the EP extract plus plant nutrients (20-20-20 N-P-K, plus micronutrients, one tablespoon per gallon of solution; Ralston-Purina, St. Louis, Mo.). There were 50 wheat seeds in each of 5 containers and 15 soybean seeds in each of 10 containers, giving a total of 250 and 150 seeds, respectively.

Sand was selected as a growth medium to eliminate potential confounding of test results by attenuation of toxicants associated with clay particles and organic matter present in natural field soils. Establishment of a more realistic soil medium capable of being universally standardized for application to such assays was deemed to be a research task in itself beyond the scope of this project.

Plants were exposed to the solution above added to sand and misted with an atomizer for leaf exposure. The dose was sufficient to restore loss by evapotranspiration. The amount of time between each application ranged from every other day to every 3 days.

Wheat plants were grown for 2 weeks and soybeans for 3 weeks. At harvest, sand was washed from the roots, roots and shoots were separated, and dry weights were recorded for 12 of the extracts. Five soybean plants and ten wheat plants were consolidated to reduce variability between samples. The N value was the number of sample groups available. A standard t-test was used for comparison of treated and control weights. The last four extracts (fluidized bed residue, municipal sewage sludge, power plant No. 2 fly ash, and power plant No. 1 treated scrubber sludge) were compared with controls by measurement of lengths of root and shoot. Measuring length was much faster, and the N value was larger since each plant represented one observation. Also, it was always possible that not all sand was completely rinsed off of the roots, which would create an error in final weight; length measurements eliminate this problem.

APPENDIX G

SALMONELLA MUTAGENICITY ASSAY

The realization that the list of potential chemical carcinogens is growing faster than our capacity to test the materials and the enormous increase in industrial and technological activities have created an interest in short-term test procedures for the identification of genetic hazards associated with environmental chemical pollutants. Although the health effects of chemicals in the environment are being extensively studied, it is obvious that short-term test procedures are necessary to reduce the study time for evaluating the large number of potentially hazardous substances. To control the problem of environmental carcinogenesis, greater numbers of these compounds are to be screened and assigned priorities for further testing. This appears to be the primary role of the short-term test. Not only should a meaningful short-term test be faster, easier to interpret, more sensitive, and less expensive, but it must also be reliable and relevant to the in vivo assays.

Among the various short-term assays which utilize microbial organisms, the Salmonella test system developed by Ames has been widely used as a prescreen for the determination of genetic and potential carcinogenic hazards of complex environmental effluents or products. This test system has been examined more extensively than any other short-term assay for correlating mutagenicity and carcinogenicity.[1] It utilizes a series of histidine-requiring mutants that revert after treatment with mutagens to the wild-type state (histidine independent). Generalized testing of the compound is accomplished by use of three strains (TA1537, TA1538, and TA98) that detect frameshift mutagens and two strains (TA1535 and TA100) that detect base-pair substitution mutagens. The design of the test is shown in Figure G-1. A recommended protocol outlining the preparation of the components of this test has been published by Ames et al.[2] Some chemicals like dimethylnitrosamine, certain hydrazines, and volatile liquids which are not mutagenic in the standard plate assays are active in the modified procedure, designated the preincubation technique. This modified procedure detects not only these compounds, but also the majority of the compounds that have been shown to be active in the standard plate assay.

BACTERIAL STRAINS

Four Salmonella typhimurium indicator strains, TA1535, TA1537, TA98, and TA100, are recommended for screening purposes. TA1535 and TA100 have base-pair substitution mutation in the histidine operon; TA100 also contains an R factor which renders the strain more sensitive to certain mutagens, possibly through error-prone repair. TA1537 and TA98 have frameshift mutation in the histidine operon; TA98 contains an R factor and is more sensitive than TA1538. TA1537 is recommended because of its unique sensitivity to some agents like 9-aminoacridine and certain ICR compounds. The characteristics of these strains are shown in Table G-1.

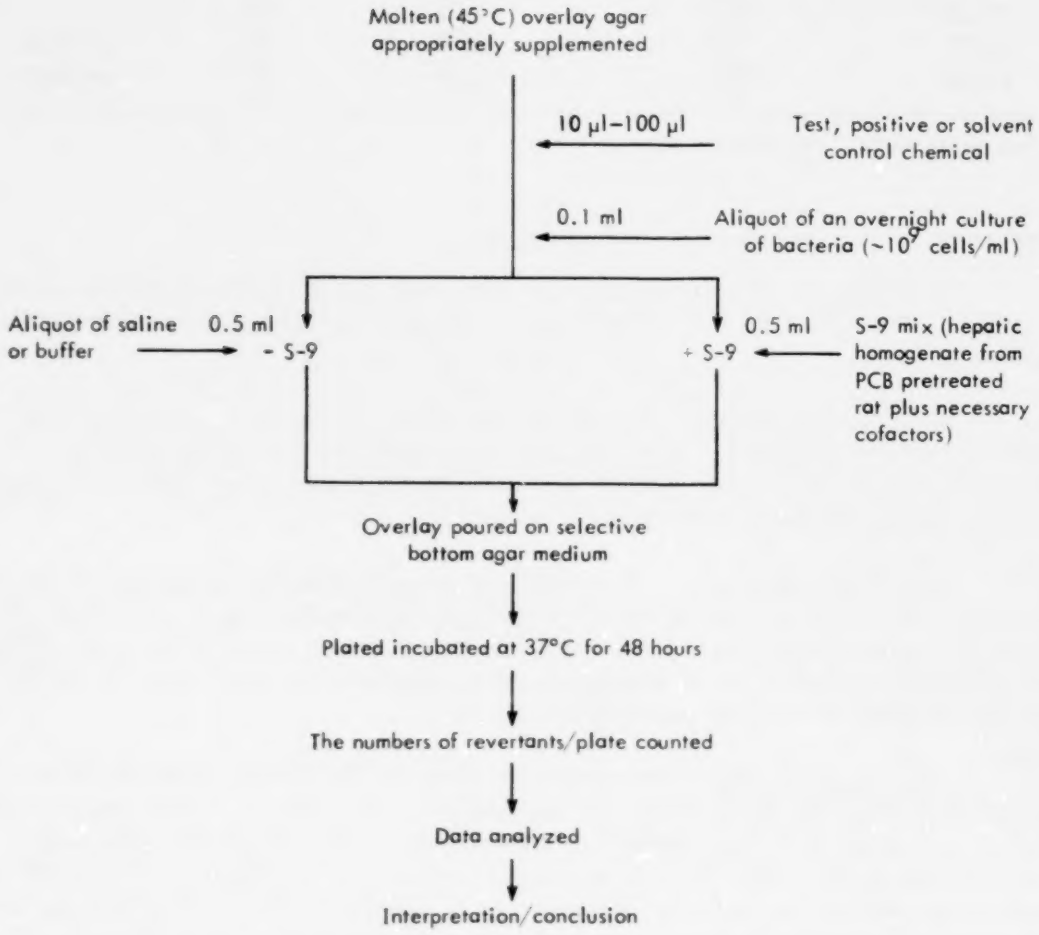
Molten (45°C) overlay agar appropriately supplemented
10 µl–100 µl
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria (~10^9 cells/ml)
Aliquot of saline or buffer
0.5 ml
- S-9
+ S-9
0.5 ml
S-9 mix (hepatic homogenate from PCB pretreated rat plus necessary cofactors)
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/conclusion

Figure G-1. Reverse mutation assay (agar incorporation method).

TABLE G-1. PROPOSED BACTERIA STRAINS*

Strain designation	Gene affected	Additional mutations		
		Repair	LPS	R factor
TA1535	hisG	uvrB	rfa	—
TA1537	hisC	uvrB	rfa	—
TA98	hisD	uvrB	rfa	pKM101
TA100	hisG	uvrB	rfa	pKM101

*See Ames et al.[1] for references.

STORAGE AND CHECKING OF TESTER STRAINS

All strains are initially grown in nutrient broth (8 g Difco-Bacto nutrient broth, 5 g NaCl/l) at 37°C for 16 h. The strains are checked for the genetic markers in the following ways:

Histidine Requirement. Streak the cultures on minimal plates both with and without histidine (spread 0.1 ml of sterile 0.1 M L-histidine on the agar surface). Biotin (0.1 ml of 0.5 mM per plate) is also essential for these strains. The strains should grow on plates containing both histidine and biotin.

Deep Rough Character. A sterile filter paper disc containing crystal violet (10 µl of 1 mg/ml) is placed on a nutrient agar petri dish containing 0.1 ml (about 10^8 bacteria) of the nutrient broth culture to be tested in a thin overlay of top agar. After 12 h incubation at 37°C, a clear zone of inhibition around the disc (about 14- to 18-mm diameter) indicates the presence of rfa mutation.

Presence of Plasmid. The strains with R factor (TA100 and TA98) should be checked routinely for the presence of the ampicillin resistance. Streak a small amount (10 µl of 8 mg/ml in 0.02 N NaOH) of an ampicillin solution across the surface of a nutrient agar plate. After the streak is dry, cultures to be checked are cross-streaked against the ampicillin, and after incubation for 12-24 h at 37°C, strains which do not contain the R factor will show a zone of growth inhibition around the ampicillin streak, whereas strains containing R factors will not.

Storage. Frozen permanent cultures containing fresh nutrient broth cultures (0.8 ml) with dimethylsulfoxide (0.07 ml) are prepared and maintained in a Revco freezer at -80°C. A working source of these cultures is maintained on master plates which are prepared as follows:

0.1 ml of sterile 0.1 M L-histidine is spread on the surface of a minimal glucose agar plate. After the histidine solution is absorbed by the agar, 0.1 ml of sterile 0.5 mM biotin is added in the same way. For TA98 and TA100, 0.1 ml of an 8 mg/ml ampicillin solution (in 0.02 N NaOH) is added. By use of a sterile loop, nutrient broth culture of the tester strain is streaked across the agar (for TA98 and TA100, plates with ampicillin are used) and incubated at 37°C for 24 h. These master plates with the cultures are stored at 4°C and can be used for several months to grow working cultures.

PREPARATION OF RAT LIVER S-9

Male Sprague-Dawley rats (of about 180-200 g weight) are given a single intraperitoneal injection of Ar-1254 at a dosage of 500 mg/kg (vehicle, corn oil) 5 days before they are killed. They are fasted 12 h before they are decapitated and allowed to bleed. The livers are aseptically removed and washed in cold 0.15 M KCl. All steps are performed at 0 to 4°C with cold and sterile solutions and glassware. The livers are minced with sterile scissors in three volumes of 0.15 M KCl (3 ml/g wet liver) and homogenized with a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for 10 min at 9000 x g, and the supernatant (S-9) is decanted and stored in convenient aliquots at -80°C. For S-9 from φB-induced rat livers, the same procedure as described above is followed except that the rats are given 0.1% sodium phenobarbital in drinking water for 1 week before they are killed.

MEDIA

Top agar (0.6% Difco-Bacto agar, 0.5% NaCl) is autoclaved and stored in 100-ml bottles at room temperature. Before use, the agar is melted (in an autoclave or in a steam bath), and 10 ml of a sterile solution of 0.05 mM L-histidine-HCl, 0.5 mM biotin is added to the 100 ml of molten agar and mixed thoroughly.

Complete medium (23.5 g BBL standard methods agar in 1 l of distilled H_2O) is autoclaved and dispensed into 100 x 15 mm plastic petri plates (30 ml/plate).

Vogel-Bonner[3] medium E with 2% glucose and 1.5% Bacto-Difco agar is used as the minimal medium for mutagenesis assays and is prepared as follows:

Vogel-Bonner Salts (50X)

Warm distilled water	670 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4 \cdot 4H_2O$)	175 g

The above salts are added to the warm water (45°C) in the specified order. Each salt is dissolved completely before the next is added. When the salts are all dissolved, the solution is cooled to room temperature. About 5 ml of chloroform is added to the solution and stored in a capped bottle at room temperature.

Dissolve 15 g of Difco-Bacto agar in 1 l of water by autoclaving. Cool to about 60 to 70°C and add 20 ml of 50 x Vogel-Bonner salt solution and 50 ml of sterile 40% glucose solution. Mix thoroughly, and dispense into 100 x 15 mm plastic petri plates (30 ml/plate). Other minimal media would presumably also serve the purpose.

PREPARATION OF S-9 MIX (ACTIVATION SYSTEM)

The S-9 mix contains the materials shown in Table G-2.

TABLE G-2. COMPOSITION OF S-9 MIX

Component*	Stock preparation	Volume (µl) of stock added/ ml of final mix	Final concentration of component in mix (µmol/ml)
1. NADP	0.1 M	40	4
2. Glucose-6-phosphate	0.1 M	5	5
3. Sodium phosphate buffer (pH 7.4)	0.2 M	500	100
4. $MgCl_2$	0.4 M	20	8
5. KCl	1.65 M	20	33
6. Homogenate	standard KCl 9000 x g supernatant	100	approx. 25 mg of fresh tissue equivalent

*Components 1 and 2 are prepared in sterile distilled water and filter-sterilized before use. Components 3–5 are prepared in distilled water, sterilized, and maintained at 4°C. Component 6 is prepared in 0.15 M KCl and stored at -80°C until used.

POSITIVE CONTROL COMPOUNDS

Any assay performed should have a control in which the solvent or diluent is employed to see its effect on the rate of spontaneous revertants. In addition to this control, a known directly acting mutagen and the one that requires metabolic activation should be used to show that the assay system is working.

The positive control compounds shown in Table G-3 could be used in these assays.

TABLE G-3. POSITIVE CONTROL COMPOUNDS

	Concentration (μg/plate)	Activation	Response of strain		
			TA1535, TA100	TA98	TA1537
Sodium azide	2.5	-	+	-*	-*
9-Aminoacridine	10.0	-	-	-	+
2-Anthramine	5.0	+	+	+	+

*Weak responses may be obtained.

MUTAGENESIS ASSAY BY THE PREINCUBATION METHOD

It may be difficult to detect biological effects with the complex environmental mixtures due to (1) toxicity of the complex mixture or (2) low concentrations of the biologically active components in the complex mixture. The first problem should be dealt with by assaying the complex mixture for general toxicity towards bacterial survival before the mutagenesis assay is performed. The second problem should be dealt with at the level of concentration and fractionation of the complex mixtures. The following protocol is recommended for general toxicity.

Only one strain, TA1537, is used to determine the general toxicity range. Overnight culture in nutrient broth is diluted to obtain about 10^3 cells/ml. To the tubes containing 2 ml standard top agar are added: 0.1 ml of the diluted culture of TA1537, various amounts of the test material (the recommended levels are: 1000, 500, 100, and 10 μl/tube), and 0.5 ml of phosphate buffer, pH 7.4 (for nonactivation) or 0.5 ml of S-9 mix (for activation). The contents are mixed and poured on the surface of a bacterial complete plate. After the agar has hardened, the plates are incubated at 37°C for 48 h. Survival is compared with a control plate containing solvent but no chemical. Once the toxicity is determined, five dose levels within the 50% or greater survival part of the curve are selected for actual mutagenesis assays.

PREINCUBATION ASSAY

Four tester strains (TA1535, TA1537, TA98, and TA100) described earlier are used in the assay, and each data point is done in duplicate. The assay is conducted as follows:

To the sterile 13 x 100 mm test tubes containing 0.5 ml of the S-9 mix placed in an ice bath, an aliquot of the test compound (or positive control mutagen or solvent or diluent) and 0.1 ml of an overnight bacterial culture are added. S-9 mix should be replaced with 0.067 M phosphate buffer (pH 7.4) in nonactivation tests. The contents are mixed and the tubes are incubated at 37° C in a shaker for 20 min. At the end of the incubation, 2 ml of molten top agar (kept at 45°C) are added per tube and the contents are gently mixed. The contents are then poured onto the surface of a Vogel-Bonner minimal glucose agar plate (appropriately labeled). After the agar has solidified, the plates are incubated at 37°C for 2 days and the his^+ revertants are recorded. Table G-4 shows the results for 2-aminoanthracene, sodium azide, and dimethylnitrosamine tested by the standard plate incorporation method and the preincubation method.

REVERTANT CONFIRMATION

Randomly selected Salmonella revertants should be picked from plates showing mutagenicity and confirmed for histidine independence by restreaking on minimal plates containing no histidine.

REPEAT TESTS

The test on each sample should be repeated within 2 weeks following the initial evaluation to confirm the results. The positive results obtained in the initial evaluation with or without PCB-induced rat liver S-9 are to be confirmed in the repeat test. If the results are negative in the initial evaluation in the presence or absence of PCB-induced rat liver S-9, it is suggested that in the repeat tests φB-induced rat liver S-9 be included in addition to the PCB-induced rat liver S-9. (It should be noted here that the liver from Ar-induced rats is the most efficient for detecting different classes of carcinogens. The liver from φB-induced rats is more efficient for detection of 2-acetylaminofluorene and many other aromatic amines, but it is very inefficient for detection of certain PAHs.) If the repeat test results are positive in the presence of φB-induced rat liver S-9, they should be reconfirmed by testing the material in the presence of φB-induced rat liver S-9 only. If the repeat test results are negative, no further testing is necessary. Figure G-2 gives the general scheme for evaluating the test material in the preincubation assay for four Salmonella tester strains.

REFERENCES FOR APPENDIX G

1. McCann, J. E., E. Choi, E. Yamasaki, and B. N. Ames. Detection of Carcinogens as Mutagens in the Salmonella/Microsome Test: Assay of 300 Chemicals. Proc. Natl. Acad. Sci. USA, 72: 5135, 1975.
2. Ames, B. N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutat. Res., 31: 347, 1975.
3. Vogel, H. J., and D. M. Bonner. Acetylornithinase of Escherichia coli: Partial Purification and Some Properties. J. Biol. Chem., 218: 97, 1956.

TABLE G-4. MUTAGENICITY OF 2-ANTHRAMINE, SODIUM AZIDE, AND DIMETHYLNITROSAMINE

Concentration of mutagen	Mutagenicity (revertants/plate) in strain					
	TA1535*		TA98		TA100*	
	Plate incorporation	Preincubation	Plate incorporation	Preincubation	Plate incorporation	Preincubation
2-Anthramine (μg)						
0			36	45		
0.01			66	78		
0.1			431	841		
1.0			1,994	2,104		
10.0			1,903	2,207		
Sodium azide (μg)						
0	22	15			123	113
1.0	300	274			600	1,344
2.5	566	505			865	1,902
5.0	867	765			1,065	2,372
10.0	1,643	1,100			1,275	2,746
20.0	2,063	1,456			1,336	3,015
50.0	2,260	1,675			1,362	3,161
Dimethylnitrosamine (μl)						
0	15	29			113	161
1.0	22	35			176	164
2.5	23	35			182	164
5.0	28	41			176	173
10.0	20	349			178	516
20.0	25	583			209	1,090
50.0	2	24			0	122

*Mean of six replicate runs.

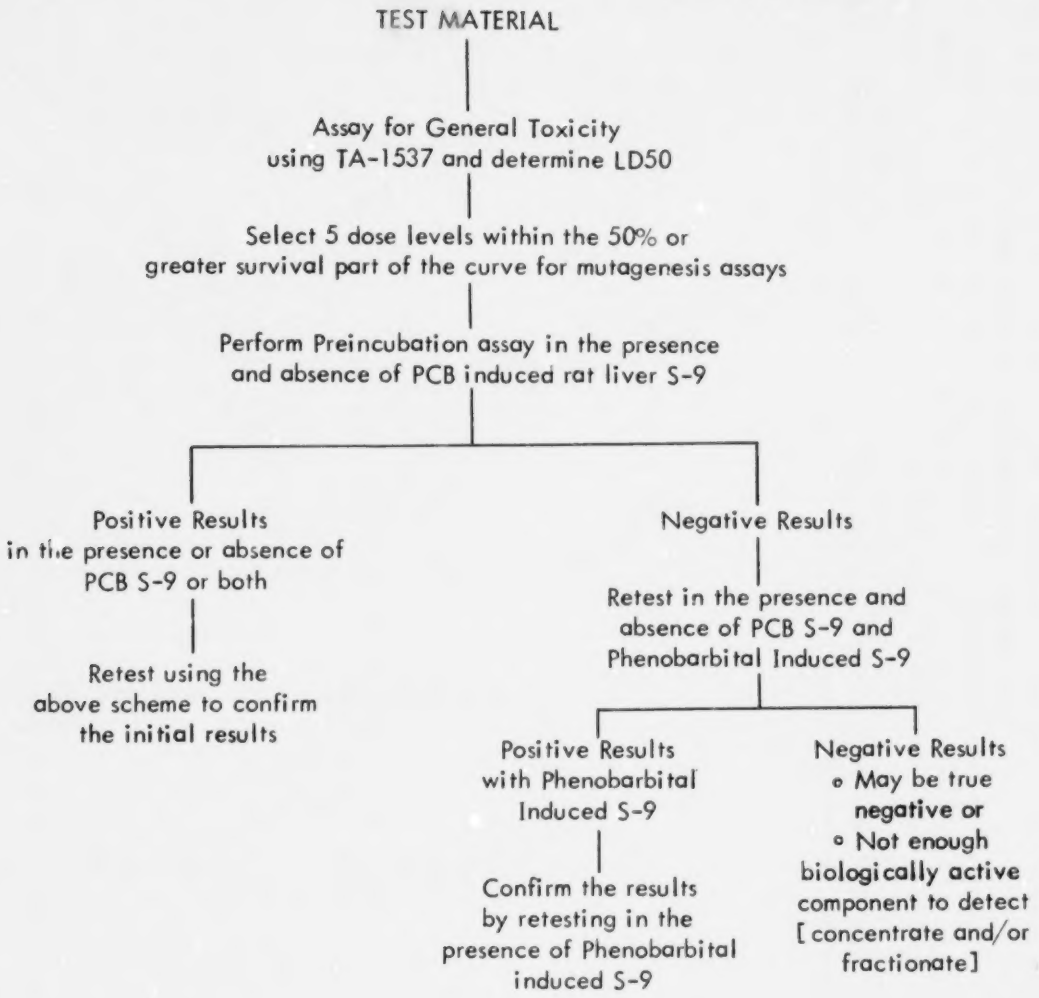
TEST MATERIAL
Assay for General Toxicity using TA-1537 and determine LD50
Select 5 dose levels within the 50% or greater survival part of the curve for mutagenesis assays
Perform Preincubation assay in the presence and absence of PCB induced rat liver S-9
Positive Results in the presence or absence of PCB S-9 or both
Retest using the above scheme to confirm the initial results
Negative Results
Retest in the presence and absence of PCB S-9 and Phenobarbital Induced S-9
Positive Results with Phenobarbital Induced S-9
Confirm the results by retesting in the presence of Phenobarbital induced S-9
Negative Results
o May be true negative or
o Not enough biologically active component to detect [concentrate and/or fractionate]

Figure G-2. General scheme.

APPENDIX H

SACCHAROMYCES CEREVISIAE GENE MUTATION ASSAY

Both forward and reverse mutation can be monitored in the haploid strain XL7-10B.[1] It has the genotype $\alpha\ \rho^+$ CAN1 his1-7 lys1-1 ura1.

FORWARD MUTATION TO CANAVANINE RESISTANCE (CAN1 → can1)

Canavanine is a toxic arginine analog to which yeast is normally sensitive. Resistance to canavanine has been shown to be almost exclusively due to mutational inactivation of the arginine permease. The permease gene (CAN1) has been estimated to be approximately 7700 nucleotides long, hence it offers a very large mutational 'target.' CAN1 is mutated by both frameshift and base-pair substitution inducing mutagens; in addition, deletions and chromosomal rearrangements with breakpoints in CAN1 should also be recoverable.

REVERSION OF his1-7

The his1-7 mutation is a missense mutation resulting from a base-pair substitution in a histidine biosynthesis gene. This mutation confers a requirement for the amino acid histidine. Back mutation by base-pair substitution at the original mutant site removes the histidine requirement. Further, his1-7 reverts by second site mutation — a second base-pair substitution at another site which 'corrects' the original amino acid replacement in the enzyme protein by a second compensatory replacement. Since the reversion event is not limited to a single site, a broader spectrum of base-pair substitutions can be detected. Also, owing to different modes of DNA repair in yeast, his1-7 is reverted by mutagens which have been classified in bacterial systems as acting via a frameshift mechanism.

Both CAN1 and his1-7 mutate readily, and the mutants are subject to a positive selection method. Additionally, this system will tolerate a wide variety of assay conditions (e.g., stationary phase versus log phase cells or presence or absence of a mammalian microsomal activation system) without requiring modification of the mutant selection procedure or affecting the recovery of mutants.

SUPPLIES AND EQUIPMENT

YPD, SC-ARG+CAN, and SC-HIS agar plates, prepoured
Sterile solution of 0.067 M K_2HPO_4
Sterile solution of 10% (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes
Sterile plastic test tubes with sealing caps (16 x 100 mm is convenient - available from Falcon)
Shaking water bath set at 30°C (rotary preferred)
A supply of sterile 10-, 5-, and 1-ml pipettes and tips for microliter pipetor

Sufficient S-9 mix[2] for activated assays (prepare fresh and hold on ice, maximum 3 h)
Ice bath for stopping assay
Sterile 0.067 M K_2HPO_4 dilution blanks (in plastic tubes as above).
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or dimethylsulfoxide solution
Hemocytometer and compound microscope

MEDIA

Media have the compositions shown below and are sterilized by autoclaving.

YPD

1% Difco yeast extract	6 g
2% Difco-Bacto-peptone	12 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

For broth leave out agar.

SD

0.67 % Difco yeast nitrogen base without amino acid	4 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

A modified synthetic complete is prepared by the following additions to SD (concentrations in mg/l).

SC

adenine sulfate	20
uracil	20
L-tryptophan	20
L-histidine HCl	20
L-arginine HCl	20
L-methionine	20
L-leucine	30
L-lysine HCl	30

SC-ARG+CAN is prepared by deleting arginine and adding filter-sterilized canavanine sulfate (40 mg/l) after autoclaving. SC-HIS is prepared by deleting histidine.

ASSAY METHODOLOGY

Suspend a well-formed isolated colony of the appropriate tester strain in 0.067 M K_2HPO_4 and determine the cell concentration using the hemocytometer. Prepare a dilution series and inoculate 25 ml of YPD broth with approximately 200 cells. Grow 2-3 days with vigorous shaking at 30°C until late stationary phase.

Centrifuge the stationary-phase culture and resuspend in buffer. Adjust cell concentration to 2×10^9 cells/ml.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml of aqueous test material (or up to 100 µl of dimethyl sulfoxide solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 M K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally, add 0.1 ml of the yeast suspension to each assay tube. Seal the caps.

Without delay, place the assay tubes in the 30°C shaking water bath. At least a 3-h and a 20-h incubation should be performed.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10% $Na_2S_2O_3$ to each tube.

PLATING

Plate the stopped incubation mixture directly on SC-ARG+CAN and SC-HIS (in duplicate, 0.1 ml/plate).

Dilute the stopped mixture 10^{-5} and plate on YPD to determine survival.

Spread to dryness, flaming the spreader for each plate.

Incubate YPD plates 3 days at 30°C, others 5 days.

Count the plates. Calculate the percent survival and the mutation frequency based on surviving titer and note the mutation yield.

NOTES

Activation: φB-induced and Ar- (or substitute) induced S-9 are used, as per Ames.[2]

Enzyme titration: after the dose giving 50% survival, or the highest dose applicable (if the substance is nontoxic), is determined, the activation system is optimized by titration with varying amounts of S-9.

REFERENCES FOR APPENDIX H

1. Strain available from F. W. Larimer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.
2. Ames, B. N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutat. Res., 31: 347, 1975.

APPENDIX I

BACTERIAL DNA REPAIR ASSAY

DNA repair tests do not measure mutation per se, but DNA damage induced by chemical treatment of a cell. Microbial test systems measure this damage as cell killing. Test systems employ paired, identical cells, except one has the normal DNA repair capabilities and one lacks a specific step (or steps) in the enzyme pathways responsible for DNA repair. Preferential killing of the repair-deficient strain by the test chemical implies that the chemical exerts its killing effect by reacting with the cells' DNA and, therefore, may be mutagenic. This implication may not be valid in all cases, since the test cannot separate a purely lethal DNA effect from one that also has a mutagenic component.

The following protocol describes a generalized DNA repair assay which can utilize any of the major bacterial 'repair' strains, i.e., the Bacillus subtilis rec^+ - rec^- pair,[1] Escherichia coli $polA^+$-$polA^-$,[2] or Salmonella typhimurium $uvrB^+$-$uvrB^-$.[3] These systems are all based on the hypersensitivity of repair-defective bacteria to the lethal effects of DNA-modifying chemicals.

STRAIN MAINTENANCE

The source references for the strains chosen give details for the maintenance of master cultures. The repair phenotypes are conveniently verified by checking for UV sensitivity as follows:

The tester strains are parallel-streaked across individual nutrient agar plates and half of each plate is irradiated with a G.E. 15 W germicidal lamp at a distance of 33 cm. The duration of the UV exposure is 6 s, after which the plates are incubated overnight at 37°C. The repair-deficient strain should show growth only on the unirradiated side of the plate, while the repair-proficient strain should show growth on both sides of the plate.

SUPPLIES AND EQUIPMENT

Prepoured nutrient agar plates
Sterile solution of 0.067 M K_2HPO_4
Sterile solution of 10% (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes
Sterile plastic test tubes with sealing caps (16 x 100 mm is convenient — available from Falcon)
Shaking water bath set at 37°C (rotary preferred)
A supply of sterile 10-, 5-, and 1-ml pipettes and tips for microliter pipetor
Sufficient S-9 mix[3] for activated assays (prepare fresh and hold on ice, maximum 3 h)
Ice bath for stopping assay

Sterile 0.067 M K_2HPO_4 dilution blanks (in plastic tubes as above)
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or dimethylsulfoxide solution

MEDIA

Nutrient broth is composed of 8 g Difco-Bacto nutrient broth, 5 g NaCl, and distilled water to 1 l; sterilization is by autoclaving. Nutrient agar is nutrient broth solidified with 2% Difco-Bacto agar.

REPAIR ASSAY

Prepare overnight at 37°C nutrient broth cultures of each tester strain; store at 4°C.

0.1 ml of each bacterial culture will be required for each respective assay point. Centrifuge on adequate volume of each culture, discard the broth supernatant, and resuspend the bacteria in a like volume of 0.067 M K_2HPO_4.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml of aqueous test material (or up to 50 µl of dimethylsulfoxide solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 M K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally, add 0.1 ml of the appropriate bacterial suspension to each of the assay tubes. Seal the caps.

Without delay, place the assay tubes in the 37°C shaking water bath. Incubate unactivated assays for 20 min, activated assays for 2 h.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10% $Na_2S_2O_3$ to each tube.

PLATING

Prepare the following serial dilutions from each stopped assay tube: 1:100, 1:10, 1:10, 1:10, using the 0.067 M K_2HPO_4 dilution blanks.

For each dilution, pipet 0.1 ml onto duplicate nutrient agar plates. Spread to dryness, flaming the spreader for each plate.

Incubate the plates inverted at 37°C overnight.

Count the plates. Calculate percent survival for each strain at each assay point, relative to untreated controls.

REFERENCES FOR APPENDIX I

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